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Regulation of 11β-hydroxysteroid dehydrogenase type 2 by steroid hormones and epidermal growth factor in the Ishikawa human endometrial cell line

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Abstract

The biological actions of glucocorticoids in target organs are determined at least in part by the local expression of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which is responsible for the inactivation of glucocorticoids. The human endometrium is a glucocorticoid target tissue, and is known to express 11β-HSD2. However, little is known about the function and regulation of 11β-HSD2 in the endometrium, probably owing to the lack of in vitro model systems (i.e., cell lines) that express 11β-HSD2. Here, we describe the characterization of 11β-HSD expression in Ishikawa cells, a well-differentiated human endometrial adenocarcinoma cell line. The 11β-HSD activity in intact Ishikawa cells was characteristic of 11β-HSD2 in that it only possessed dehydrogenase activity (cortisol to cortisone) and had a high affinity for cortisol (apparent K_m of 34 nM). The exclusive expression of 11β-HSD2 in Ishikawa cells was confirmed by RT-PCR which demonstrated the presence of the mRNA for 11β-HSD2 but not that for 11β-HSD1.

To investigate the regulation of 11β-HSD2 in Ishikawa cells, we treated these cells with sex steroid hormones, glucocorticoids and epidermal growth factor (EGF), and determined the effects of these treatments on 11β-HSD2 activity by an established intact cell radiometric conversion assay. Treatment with estradiol-17β (E_2, 10 nM) and medroxyprogesterone acetate (MPA, 100 nM) produced a classic sex steroid effect; the greatest increase (330% of the control) in the level of 11β-HSD2 activity was caused by the combined treatment, followed by MPA (240% of the control) with E_2 being the least effective (156% of the control). The stimulatory effect of E_2 was blocked by the pure antiestrogen ICI 182,780. The synthetic glucocorticoid dexamethasone (Dex) increased 11β-HSD2 activity in a time- and dose-dependent manner (200% of the control; 100 nM for 48 h), and the endogenous glucocorticoid cortisol was equally effective in this regard. The antiprogesterone-antiglucocorticoid RU486 did not counteract with MPA or Dex but rather acted as an agonist; increased 11β-HSD2 activity (160% of the control; 100 nM for 72 h). By contrast, treatment with EGF caused a dose- and time-dependent decrease in 11β-HSD2 activity (60% of the control; 10 ng/ml for 72 h). In addition, semi-quantitative RT-PCR analysis revealed that there were corresponding changes in the level of 11β-HSD2 mRNA following the treatment of Ishikawa cells with these steroid hormones and EGF, indicating that the effects of these hormones and EGF are mediated, at least in part, at the level of 11β-HSD2 gene transcription. In conclusion, we have demonstrated for the first time that the human Ishikawa endometrial cell line expresses exclusively the 11β-HSD2 isozyme. Moreover, we have presented the first direct evidence that sex steroid hormones and glucocorticoids stimulate while EGF inhibit the expression of 11β-HSD2 in Ishikawa cells, suggesting that endometrial 11β-HSD2 is under the control of steroid hormones and EGF. Thus, the Ishikawa cell line represents an excellent model in which the function and regulation of endometrial 11β-HSD2 may be studied.

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1. Introduction

In mammalian uterus, glucocorticoids exert potent effects, largely in an inhibitory fashion, on a number of dynamic functions, including cellular proliferation [1], apoptosis [2,3], and synthesis of hormones, growth factors and enzymes [4–6]. Recently, there is increasing evidence that glucocorticoid actions in target tissues are controlled not only by the function of glucocorticoid receptors (GRs) but also by the local expression of 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes [7,8], which determine the intracellular level of bioactive glucocorticoids.

To date, two distinct isozymes of 11β-HSD, known as 11β-HSD1 and 2, have been identified, cloned and characterized [7]. 11β-HSD1 is a low affinity NADP(H)-dependent dehydrogenase (cortisol to cortisone)/reductase (cortisone to cortisol) enzyme, with an apparent K_m for glucocorticoids in the μM range [9,10]. This enzyme is widely expressed in glucocorticoid target tissues, most notably the liver [11,12]. In intact cells, 11β-HSD1 functions predominantly as a reductase generating cortisol from cortisone which circulates largely in the free, unbound form [13]. Although it has been proposed that 11β-HSD1 regulates the intracellular level of bioactive glucocorticoids, the precise physiological role of this enzyme in individual target sites remains obscure.

In contrast, 11β-HSD2 is a high affinity NAD-dependent unidirectional dehydrogenase enzyme, with an apparent K_m for glucocorticoids in the nM range [14–17]. The expression of this isozyme is restricted to the placenta where it is proposed to serve as a barrier to protect the fetus from high levels of maternal glucocorticoids [18,19], and to aldosterone-target organs such as the kidney [15] where it protects the non-selective mineralocorticoid receptors from high circulating levels of glucocorticoids [20,21]. Deficiencies in this enzyme, either congenital (caused by mutations in the gene encoding 11β-HSD2) or acquired (through liquorice ingestion), leads to the syndrome of apparent mineralocorticoid excess (AME) in which cortisol acts as a mineralocorticoid causing hypertension and hypokalemia [22–24].

Given the wide range of glucocorticoid effects in mammalian uterus, local expression of 11β-HSD enzymes will likely play an important role in the function of the uterus. In a recent study, Smith and colleagues [25] showed that 11β-HSD2 immunoreactivity was localized in luminal and glandular epithelia of the human endometrium. Furthermore, the level of 11β-HSD2-like activity in endometrial tissue homogenates was higher in the secretory than in the proliferative phase, suggesting that endometrial 11β-HSD2 is under the control of sex steroid hormones. Currently few, if any, models exist for the study of human endometrial 11β-HSD2.

The Ishikawa human endometrial adenocarcinoma cell line has been used extensively as a model to study endometrial function since this cell line expresses many of the same enzymes, steroid hormone receptors, growth factors and their receptors as well as structural proteins found in normal endometrium [26,27]. Therefore, the present study was undertaken to determine whether Ishikawa cells express the 11β-HSD2 isozyme, and if so, whether its expression is regulated by steroid hormones. Given that epidermal growth factor (EGF) is produced and functions locally within the human endometrium [28], we also examined the effects of EGF on 11β-HSD2 expression in Ishikawa cells.

2. Materials and methods

2.1. Reagents and supplies

[1,2,6,7-^{3}H(N)]-Cortisol (80 Ci/mmol) was purchased from Du Pont Canada Inc. (Markham, Ontario). Non-radioactive steroids were obtained from Steraloids Inc. (Wilton, NH). Dexamethasone, estradiol-17β and medroxyprogesterone acetate were obtained from Sigma Chemicals (St. Louis, MO). Recombinant human epidermal growth factor was obtained from R&D Systems (Minneapolis, MN). Polyester-backed thin-layer chromatography (TLC) plates were obtained from Fisher Scientific Ltd (Unionville, Ontario). All solvents used were OmniSolv grade from BDH Inc. (Toronto, Ontario). General molecular biology reagents were from Gibco BRL (Burlington, Ontario) or Pharmacia Canada Inc. (Baie D'Urte, Quebec). The cDNAs used in this study were labelled with [^{32}P]dCTP (Du Pont Canada; 3000 Ci/mmol) by random priming. Oligonucleotides were synthesized using a Pharmacia Gene Assembler and purified using NAP-50 columns (Pharmacia) according to the manufacturer's instructions. Cell culture supplies were obtained from Gibco BRL or Fisher Scientific.

2.2. Cell culture and treatments

Ishikawa cells were kindly provided by Dr. M. Nishida (Tsukuba University, Japan). Cells were routinely grown in DMEM/F-12 (1:1) supplemented with 10% FBS, penicillin-streptomycin and sodium pyruvate. Cells were maintained in T-25 Corning flasks at 37°C in a 95% air–5% CO_2 humidified incubator. The medium was changed every other day. The cells were passed as required. To study the effects of different treatment regimes on 11β-HSD2 activity, cells were passed onto 12-well Corning plates and cultured to 60–70% confluence. The cells were then cultured in

serum-free medium 24 h prior to treatment, and all the treatments in triplicate wells were carried out under serum-free conditions. Controls (also in triplicates) were incubated similarly but without the addition of treatment. For 11β-HSD2 mRNA analysis, cells were subjected to identical culture and treatment conditions as described above except that they were maintained in T-25 flasks.

2.3. Assay of 11β-HSD2 activity — radiometric conversion assay

The level of 11β-HSD2 activity in intact cells was determined by measuring the rate of cortisol to cortisone conversion, as described previously [29]. Briefly, at the end of treatment, the cells were washed 3 times in serum-free medium to remove the compounds, in an attempt to exclude their having a possible competitive inhibition. The cells were then incubated for 4 h at 37°C in serum-free medium containing approx. 100,000 cpm [^{3}H]cortisol and 10 nM unlabelled cortisol. At the end of incubation, the medium was collected, and steroids extracted. The extracts were dried, and the residues resuspended. A fraction of the resuspension was spotted on a TLC plate which was developed in chloroform/methanol (9:1, v/v). The bands containing the labelled cortisol and cortisone were identified by UV light of the cold carriers, cut out into scintillation vials and counted in Scintisafe^{TM} Econol 1 (Fisher Scientific, Toronto, Canada). The rate of cortisol to cortisone conversion was calculated from the radioactivity of cortisol and cortisone, and the blank values (defined as the rate of conversion in the absence of cells) were subtracted, and the results expressed as the percentage conversion. Data are presented as mean $\pm$ SEM of 3–6 independent experiments.

2.4. Analysis of 11β-HSD2 mRNA — semi-quantitative RT-PCR

To determine if changes in 11β-HSD2 activity following the different treatment regimes were associated with alterations in 11β-HSD2 mRNA, the relative abundance of 11β-HSD2 mRNA in Ishikawa cells was assessed by an established semi-quantitative RT-PCR protocol, as described [29]. Briefly, total RNA was extracted from cultured cells using RNeasy kit (QIAGEN Inc., Mississauga, Ontario, Canada) according to the manufacturer's instructions. Prior to use, the RNA samples (1–2 μg) were checked by agarose gel electrophoresis in the presence of formaldehyde, and the integrity of the RNA was assessed by the presence of two sharp bands representing 28 S and 18 S rRNA after staining with ethidium bromide. One microgram of total RNA was reverse-transcribed using a standard oligo-dT primer in a total volume of 20 μl. An aliquot (2 μl) of the RT reaction products was then subjected to a standard PCR (95°C, 55 s; 55°C, 55 s; 72°C, 1 min; 28 cycles) using sequence-specific primers (forward primer, 5′-AGTAGTTGCTGATGCGGA; reverse primer, 5′-CATGCAAGTGCTCGATGT) which correspond to nucleotides 624–641 and 1004–1021 in the published human 11β-HSD2 cDNA [15], respectively. GAPDH was used as a control. The same PCR conditions were used for GAPDH except that a cycle number of 23 instead of 28 was used. The primers for GAPDH (forward primer, 5′-ACCACAGTCCATGCCATCAC; reverse primer, 5′-TCCACCACCCTGTTGCTGTA) were obtained from Clontech Laboratories Inc. (Palo Alto, CA), and they correspond to nucleotides 586–605 and 1018–1037 in the published human GAPDH cDNA [30]. To verify the RT-PCR products and to assess the relative abundance of 11β-HSD2 mRNA, a fraction of the RT-PCR products was then subjected to a standard Southern blot analysis, using [^{32}P]-human 11β-HSD2 cDNA and [^{32}P]-human GAPDH cDNA as probes.

To determine the relative abundance of 11β-HSD2 mRNA and GAPDH mRNA, the relative optical density of the corresponding signals on autoradiographic films were measured by scanning with a laser densitometer (LKB 2222-020 UtraScan XL; LKB Produkter AB, Bromma, Sweden), as described [31]. In all cases, the signals were detected within the linear scan range of the densitometer. For each RNA sample, the ratio of 11β-HSD2 mRNA signal to GAPDH mRNA signal was calculated, and group means were obtained.

2.5. Data analysis

Statistical analyses of 11β-HSD2 mRNA and 11β-HSD2 activity data were performed using one-way ANOVA, followed by LSD (least squares difference) test. Significance was set at $p < 0.05$.

3. Results

3.1. Characterization of 11β-HSD in Ishikawa cells

Using cortisol and cortisone as physiological substrates, cultured Ishikawa cells displayed predominantly dehydrogenase activity (cortisol to cortisone) with negligible reductase activity (cortisone to cortisol) (data not shown). Furthermore, the dehydrogenase activity of 11β-HSD had a high affinity for cortisol (apparent K_m, 34.3 ± 10.7 nM; Fig. 1). Collectively, these activity data indicated the presence of 11β-HSD2 in Ishikawa cells.

To confirm the expression of 11β-HSD2 in Ishikawa cells, RT-PCR was used to examine the presence of

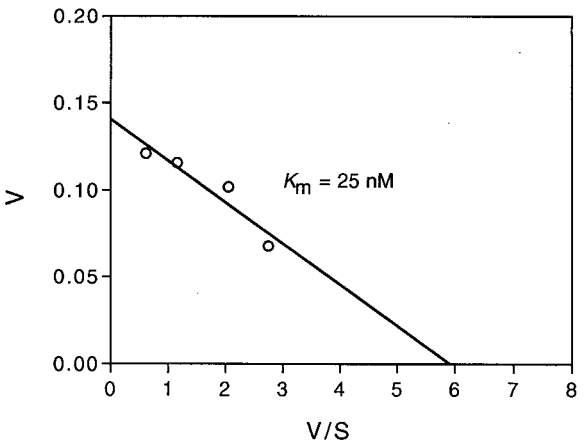

Fig. 1. Plot according to the Michaelis–Menten equation for determining kinetic parameters of 11β-HSD dehydrogenase activity. Data from a representative experiment are shown. Radiometric conversion assays were conducted using intact cells, as described in the Materials and methods, with varying amounts of cortisol (0.025–0.25 μM). V, velocity (pmol/min/well); S, substrate concentration (μM).

11β-HSD2 mRNA. It was found that the mRNA for 11β-HSD2 but not that for 11β-HSD1 was detected (data not shown). This indicated that Ishikawa cells express exclusively the 11β-HSD2 isozyme.

3.2. Effects of sex steroid hormones on 11β-HSD2 activity

To study the effects of sex steroid hormones, Ishikawa cells were treated for 72 h with 10 nM estradiol-17β (E_2), 100 nM medroxyprogesterone acetate (MPA), or 10 nM E_2 plus 100 nM MPA. This treatment regime produced a classic sex steroid effect; the greatest increase in the level of 11β-HSD2 activity was caused by the combined treatment, followed by MPA with E_2 being the least effective (Fig. 2A).

To examine the involvement of estrogen receptors (ER), Ishikawa cells were treated for 72 h with E_2 (10 nM), ICI 182,780 (100 nM), a pure antiestrogen, or the two together. While it had no effect alone, the ICI compound abolished the stimulatory effects of E_2 on 11β-HSD2 activity (Fig. 2B). However, when the cells were treated with MPA (100 nM) plus the anti-progesterone RU486 (100 nM), RU486 did not counteract with MPA, rather acted as an agonist; increased 11β-HSD2 activity (Fig. 2C).

3.3. Effects of glucocorticoids on 11β-HSD2 activity

To study the effects of glucocorticoids, Ishikawa cells were treated with 100 nM dexamethasone (Dex) for different lengths of time (24, 48 and 72 h). Dex resulted in a time-dependent increase in the level of 11β-HSD2 activity with more than 2-fold increase after 72 h treatment (Fig. 3A). When the cells were exposed to different concentrations of Dex, ranging from 1 to 1000 nM for 48 h, there was a dose dependent increase in the level of 11β-HSD2 activity with a significant stimulation at 10 nM (Fig. 3B). To examine if the endogenous glucocorticoid, cortisol, was also effective in this regard, Ishikawa cells were treated

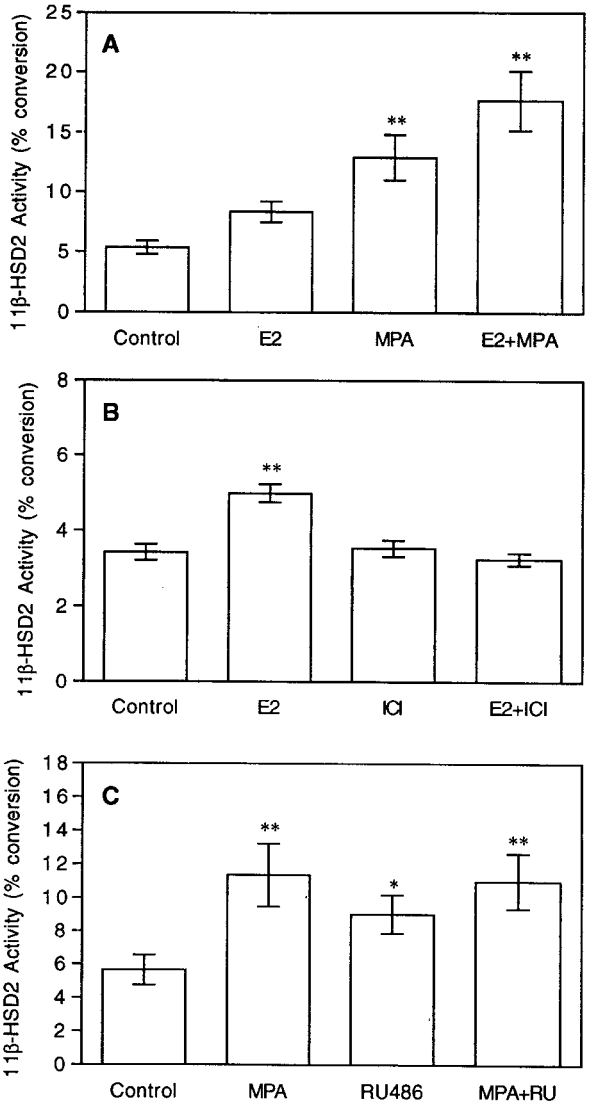

Fig. 2. Effects of estradiol and progesterone on 11β-HSD2 activity. Ishikawa cells were incubated in the absence of serum for 72 h with (A) estradiol-17β (E_2; 10 nM), medroxyprogesterone acetate (MPA; 100 nM), or E_2 (10 nM) plus MPA (100 nM); (B) estradiol-17β (E_2; 10 nM), ICI 182,780 (ICI; 100 nM), or E_2 (10 nM) plus ICI (100 nM); and (C) MPA (100 nM), RU486 (RU; 100 nM), or MPA (100 nM) plus RU (100 nM). At the end of treatment, the level of 11β-HSD2 activity in intact cells was determined by a radiometric conversion assay, as described in the Materials and methods. Bars represent the mean ± SEM of 3–6 independent experiments, each performed in triplicate. $^{*}p < 0.05$, and $^{**}p < 0.01$ when compared with the control.

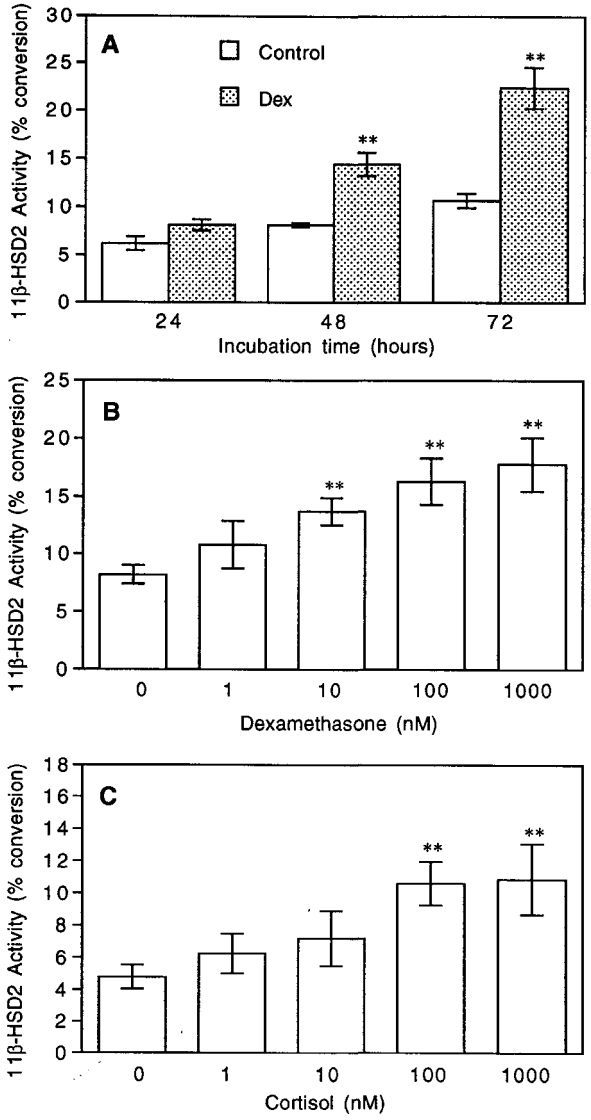

Fig. 3. Effects of glucocorticoids on 11β-HSD2 activity. Ishikawa cells were incubated in the absence of serum (A) with 100 nM dexamethasone (Dex) for the indicated times; (B) for 48 h with the indicated concentrations of Dex; and (C) for 48 h with the indicated concentrations of cortisol. At the end of treatment, the level of 11β-HSD2 activity in intact cells was determined by a radiometric conversion assay, as described in the Materials and methods. Bars represent the mean $\pm$ SEM of 3–6 independent experiments, each performed in triplicate. $^{*}p < 0.05$, and $^{**}p < 0.01$ when compared with the control.

with cortisol (1–1000 nM) for 48 h. This resulted in a dose-dependent increase in the 11β-HSD2 activity, and the magnitude of this increase was comparable with that seen after Dex treatment (Fig. 3C). Again, RU486, an anti-glucocorticoid and anti-progesterone, did not block the stimulatory effects of Dex on 11β-HSD2 activity (data not shown).

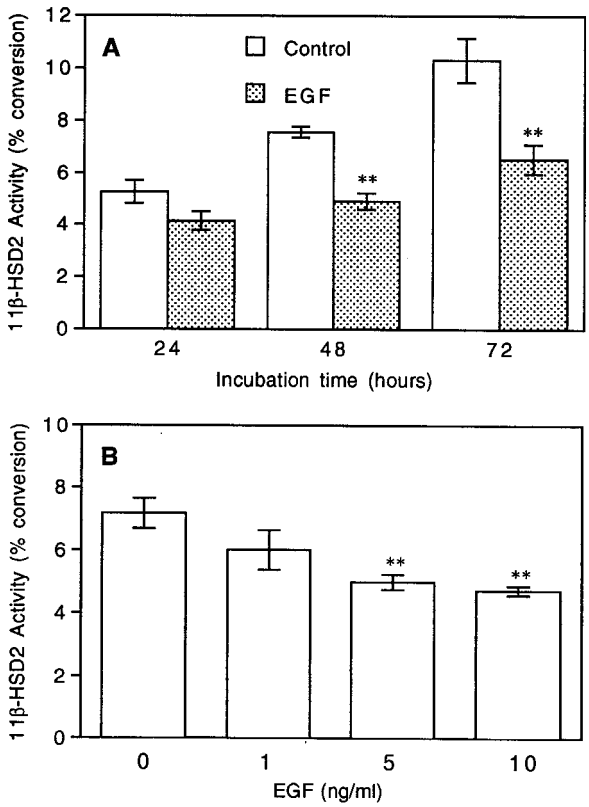

Fig. 4. Effects of epidermal growth factor (EGF) on 11β-HSD2 activity. Ishikawa cells were incubated in the absence of serum (A) with 10 ng/ml EGF for the indicated times; and (B) for 48 h with the indicated concentrations of EGF. At the end of treatment, the level of 11β-HSD2 activity in intact cells was determined by a radiometric conversion assay, as described in the Materials and methods. Bars represent the mean $\pm$ SEM of 3–6 independent experiments, each performed in triplicate. $^{*}p < 0.05$, and $^{**}p < 0.01$ when compared with the control.

3.4. Effects of EGF on 11β-HSD2 activity

To study the effects of EGF, Ishikawa cells were treated with 10 ng/ml EGF for 24–72 h, there was a time-dependent decrease in the level of 11β-HSD2 activity (Fig. 4A). When the cells were exposed to different concentrations of EGF (1, 5 and 10 ng/ml) for 48 h, there was a dose dependent inhibition of 11β-HSD2 activity with a significant and near maximal effect at 5 ng/ml (Fig. 4B).

3.5. Effects of sex steroids, glucocorticoids and EGF on 11β-HSD2 mRNA

In order to determine if changes in 11β-HSD2 activity following E_2, MPA, Dex and EGF treatment were associated with alterations in 11β-HSD2 mRNA, the relative level of 11β-HSD2 mRNA was assessed by a semi-quantitative RT-PCR protocol. As shown in

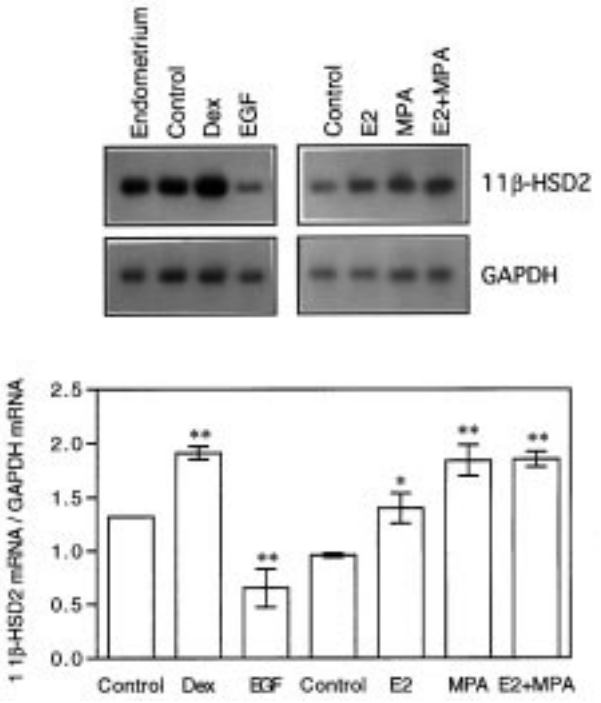

Fig. 5. Effects of steroid hormones and EGF on 11β-HSD2 mRNA. One representative autoradiograph of the semi-quantitative RT-PCR analysis of 11β-HSD2 mRNA (top panel) and changes in the relative abundance of 11β-HSD2 mRNA (bottom panel) in Ishikawa cells following different treatment regimes are shown. Cells were treated in serum free medium for 48 h with dexamethasone (Dex; 100 nM), EGF (10 ng/ml), or for 72 h with estradiol-17β (E_2; 10 nM), medroxyprogesterone acetate (MPA; 100 nM), or E_2 (10 nM) plus MPA (100 nM). At the end of treatment, total cellular RNA was extracted and subjected to a semi-quantitative RT-PCR, followed by Southern blotting, as described in the Materials and methods. Total RNA from human endometrium was used as a positive control. Bars represent the mean $\pm$ SEM of 3–6 independent experiments. $^{*}p < 0.05$, and $^{**}p < 0.01$ when compared with the control.

Fig. 5, there were corresponding changes in the level of 11β-HSD2 mRNA following each treatment.

4. Discussion

In the present study, we have demonstrated for the first time that the human Ishikawa endometrial cell line expresses exclusively the 11β-HSD2 isozyme. Moreover, we have presented the first direct evidence that sex steroid hormones and glucocorticoids stimulate while EGF inhibit the expression of 11β-HSD2 in Ishikawa cells, suggesting that endometrial 11β-HSD2 is under the control of steroid hormones and EGF. Thus, the Ishikawa cell line represents an excellent model in which the biological function and regulation of endometrial 11β-HSD2 may be studied.

The demonstration of 11β-HSD2 immunoreactivity in the luminal and glandular epithelia of human endometrium and the presence of 11β-HSD2-like enzyme activity in human endometrial tissue homogenates suggest an important role for 11β-HSD2 in the glucocorticoid-mediated effects on the dynamic function of human endometrium [25]. The level of 11β-HSD2-like enzyme activity in the human endometrium was significantly higher in the secretory than in the proliferative phase of the cycle [25], indicating a stimulatory effect of progesterone on endometrial 11β-HSD2 activity. Indeed, in primary cultures of human endometrial stromal cells in which 11β-HSD1 and 2 appeared to be co-expressed, MPA increased the level of both 11β-HSD1- and 2-like enzyme activities. While there was a corresponding increase in the level of 11β-HSD1 mRNA, possible changes in 11β-HSD2 mRNA were not studied [32].

The present study demonstrated that treatment of Ishikawa cells with E_2, MPA and E_2 plus MPA elicited a classic sex steroid response; the combined treatment resulted in the greatest increase in the level of 11β-HSD2 activity, followed by MPA and with E_2 being the least effective. Moreover, we have detected a corresponding increase in 11β-HSD2 mRNA, suggesting that the stimulatory effects of E_2 and MPA are mediated, at least in part, at the level of 11β-HSD2 gene transcription. When Ishikawa cells were co-incubated with E_2 and the pure anti-estrogen ICI 182,780, the ICI compound blocked the E_2-induced increase in 11β-HSD2 activity. This indicated that the stimulatory effects of E_2 on 11β-HSD2 activity were likely mediated through estrogen receptors. However, the antiprogestin–antiglucocorticoid RU486 did not counteract with MPA or Dex, rather acted as an agonist; stimulated 11β-HSD2 activity in Ishikawa cells. It is noteworthy that RU-486 has been shown previously in Ishikawa cells to function as a glucocorticoid agonist in inhibiting β-endorphin release [4] and suppressing CRH gene activation [33]. Thus, it is conceivable that Ishikawa cells may possess an altered GR which can be activated by RU-486. Alternatively, these cells may express mutated forms of co-activators and/or co-repressors.

Both cortisol and the synthetic glucocorticoid, Dex stimulated the activity of 11β-HSD2 in a dose-dependent manner in Ishikawa cells. Furthermore, a stimulation of 11β-HSD2 mRNA was observed when these cells were treated with Dex, suggesting that the stimulatory effects of glucocorticoids were mediated, at least in part, at the level of 11β-HSD2 gene transcription. These findings, for the first time, suggest a putative autoregulatory role of glucocorticoids in the control of their own local bioactive levels by inducing the expression of 11β-HSD2. If such a mechanism is operational in normal human endometrium, it is tempting to speculate that autoregulation of local glucocorticoid levels via 11β-HSD2 may serve to protect the endo-

metrial milieu and the implanting blastocyst from the teratogenic effects of excessive/elevated glucocorticoids.

It is known that both 11β-HSD1 and 2 are expressed in the human endometrium [25]. However, following implantation and decidualization of the endometrium, the decidua expresses only 11β-HSD1 [34,35]. Given that 11β-HSD1 functions predominantly as a reductase, it has been suggested that 11β-HSD1 in the decidua may provide the fetus with an extra-adrenal source of cortisol via the amniotic fluid [36]. Moreover, Murphy [37] suggested that cortisol generated from cortisone by the action of 11β-HSD1 in the uterus during pregnancy may also play an anti-immune role in the uterine wall. However, no studies have been conducted to address the disappearance of 11β-HSD2 from the human endometrium following decidualization.

There is accumulating evidence for the involvement of various growth factors in the implantation process and subsequent decidualization reaction. For instance, it has been suggested that EGF and related growth factors (TGFα) may play a role in rendering the endometrium receptive to embryo implantation [38]. Furthermore, EGF and its receptors are known to be expressed in the endometrium concomitantly with decidualization in humans [39]. Taken together, these findings suggest that EGF may play a very important role in the endometrium, especially at the time of implantation. In the present study, EGF was shown to inhibit the expression of 11β-HSD2 mRNA and enzyme activity in Ishikawa cells, suggesting that endometrial 11β-HSD2 is under the negative influence of EGF. Therefore, it is possible that EGF may be one of the contributing factors leading to the disappearance of 11β-HSD2 in the endometrium following implantation and decidualization in humans. Obviously, further studies are required to test this hypothesis.

Given the potent effects of glucocorticoids on the dynamic function of human endometrium and the crucial role of 11β-HSD2 in determining the intracellular level of bioactive glucocorticoids, it will be important to elucidate the underlying molecular mechanisms and interactions between these newly identified physiological regulators of 11β-HSD2.

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