Effect of long-term glucocorticoid treatment on oestradiol-induced proliferation in the uterus of ovariectomized rats

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Abstract

The aim of this study was to examine the effect of long-term treatment with glucocorticoids on the uterine response to oestradiol. Ovariectomized rats were treated with crystal triamcinolone acetonide (0.1 mg/100 g, i.m.) or saline (0.1 ml/100 g i.m.) for 29 days. Over this period five injections were administered, one per week. On the second day after the last triamcinolone injection, rats were treated with a single injection of oestradiol dipropionate (5 µg/100 g, s.c.) or vehicle (olive oil, 0.1 ml/100 g, s.c.). The effects of oestradiol in the uterus were determined by measuring mitotic index, bromodeoxyuridine (BrdU)-labelling index (BrdU was injected 2 h before the rats were killed; 2 mg/100 g, i.p.), and proliferating cell nuclear antigen (PCNA)-labelling index 24, 36 and 48 h

after the injection of oestradiol or vehicle. Long-term treatment with glucocorticoids resulted in dissimilar changes in oestradiol-induced proliferation in epithelial and connective-tissue (stroma) components of the uterus. In luminal and glandular epithelia, there was an initial reduction in proliferation at 24 h, followed by an increase at 36 h and a further reduction at 48 h after the oestradiol injection. In stromal cells of the endometrium, triamcinolone treatment caused a large constant increase in oestradiol-induced proliferation throughout the experiment. The glucocorticoid treatment had no effect on the parameters without oestradiol administration.

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Introduction

Strong activation of proliferative activity is the most important physiological effect of oestrogen hormones in the uterus. Oestrogens can also lead to the formation of endometrial carcinoma and other oestrogen-dependent diseases (Nagaoka *et al.* 1993). Therefore this powerful action of oestrogens must be strongly controlled. Many different biologically active substances are known to regulate oestrogen action on target tissues, including glucocorticoid hormones.

It is known that glucocorticoids can affect uterine response to oestrogen steroids. Almost all investigations of this process have clearly shown that glucocorticoids greatly diminish oestrogen-stimulated synthesis of DNA and activation of proliferation in the uterus (Tchernitchin *et al.* 1975, Campbell 1978, Stewart *et al.* 1983, Bigsby 1993). Moreover, the content of various factors and activity of various processes, such as ornithine decarboxylase activity (Stewart *et al.* 1983), number of oestrogen receptors (Lisk & Reuter 1976, Markaverich *et al.* 1981, Atkinson & Adams 1988, Rabin *et al.* 1990, Zamorano *et al.* 1992), blood flow (Monheit & Resnik 1981), protein synthesis (Lippe & Szego 1965, Sullivan *et al.* 1983), prostaglandin synthesis (Dey *et al.* 1982, Pakrasi *et al.* 1983, Jacobs *et al.*

1994), *c-fos* mRNA (Kirkland *et al.* 1992), insulin-like growth factor-I mRNA (Sahlin 1995), associated or mediated oestradiol-induced increase in mitotic activity in the uterus, are also inhibited by glucocorticoids. However, it should be noted that all the researches so far have only demonstrated glucocorticoid action after acute or short-term treatment, and there are no data on long-term effects of glucocorticoids on oestradiol-induced processes in the uterus.

The number of glucocorticoid receptors in tissues other than the uterus has been shown to decrease under conditions of chronic stress or after prolonged glucocorticoid administration (Pottinger 1990, Schmidt & Meyer 1994, Herman *et al.* 1995, Sapolsky 1996). In addition, long-term glucocorticoid treatment can lead to a decrease in prolactin concentration (Piroli *et al.* 1993, Taylor *et al.* 1995), which has been shown to result in decreased oestradiol-induced effects in the uterus (Gunin 1996). Therefore it can be suggested that uterine response to oestradiol under long-term treatment may differ from that after acute or short-term treatment with glucocorticoids. Therefore the aim of this work was to examine the effect of long-term treatment with glucocorticoids on the activity of oestradiol-stimulated proliferation in the uterus.

Materials and Methods

Animals

All procedures were performed in accordance with the *Guidelines on the Handling and Training of Laboratory Animals* published by the Universities Federation for Animal Welfare.

Female mature outbred white rats were maintained under constant conditions with free access to water and food. All the rats were ovariectomized under anaesthesia with ketamine and droperidol (7 mg/rat; 0.1 mg/rat, i.m.; Gedeon Richter, Budapest, Hungary).

Treatment protocol

From the fourth day after ovariectomy, rats received injections of crystal triamcinolone acetonide (single dose, 0.1 mg/100 g, i.m.; KENALOG; Bristol-Myers Squibb GmbH, Munich, Germany) or vehicle (saline; 0.1 ml/100 g, i.m.) once a week for 29 days, five injections in all. This crystal form of triamcinolone acetonide produces a prolonged (not less than 1 week) effect after i.m. injection (Mikhail *et al.* 1977, Lypko *et al.* 1983).

On the second day after the last triamcinolone or vehicle administration (33 days after ovariectomy), rats were treated with a single injection of either oestradiol dipropionate (5 µg/100 g, s.c.; Minmedprom, Rostov-Don, Russia) or vehicle (olive oil; 0.1 ml/100 g, s.c.).

There were seven rats per oestradiol-treated group, and five animals per untreated group. At 2 h before they were killed, all the rats were injected i.p. with bromodeoxyuridine (BrdU; 2 mg/100g; Sigma Chemical Co., St Louis, MO, USA) dissolved in saline.

Observations were made 24, 36 and 48 h after oestradiol or olive oil injection.

Methods

The middle segment of both uterine horns, the adrenal glands and the thymus were removed under deep ether anaesthesia. The animals were then killed by exsanguination.

Animals were weighed on the day of ovariectomy and just before their death. Adrenal glands and thymus were also weighed immediately after their removal. This procedure was used to control general glucocorticoid effects.

Uteri were immediately placed in fixing fluids. Part of the tissue from each animal was fixed in Carnoy's fluid (2 h at room temperature) and part in Bouin's fluid (8 h at room temperature) (Lacey 1989). All material was then dehydrated and embedded in paraffin. Transverse sections were cut at 5–7 µm.

Effects of oestradiol in the uterus were assessed using the mitotic index, the BrdU-labelling index and the proliferating cell nuclear antigen (PCNA)-labelling index.

Immunohistochemistry

BrdU and PCNA were detected using immunohistochemical staining. For determination of PCNA, sections fixed in Bouin's fluid were used, and, for determination of BrdU, sections fixed in Carnoy's fluid were used. Sections were hydrated. For detection of BrdU, slides were put in 2 M HCl for 15 min at 60 °C to denature DNA. The sections were then rinsed (3 × 10 min) in Tris-buffered saline (TBS) (pH 7.2–7.6). Further non-specific binding was blocked with 5% normal goat serum in TBS for 1 h. For PCNA detection, tissues were incubated for 2 h with primary anti-PCNA mouse monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:50 in TBS with 1% normal goat serum and 0.1% Triton X-100. For BrdU detection, sections were incubated overnight with primary anti-BrdU mouse monoclonal antibody (Sigma Chemical Co.) diluted 1:50 in TBS with 1% normal goat serum and 0.1% Triton X-100. After being rinsed in TBS (3 × 5 min), sections were incubated for 1 h with secondary goat anti-mouse immunoglobulin G antibody conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted 1:100 in TBS with 0.1% Triton X-100; the sections were then rinsed again in TBS (3 × 5 min). Alkaline phosphatase activity was revealed through the use of naphthol AS-BI-phosphate and new fuchsin as chromogens. Slides were then prepared for light microscopy. All procedures were performed at room temperature. Control sections were stained in a similar manner, except that the primary antibody was replaced with normal goat serum. In both cases, positive staining by primary antibody was indicated by a red precipitate localized in the nuclei.

Determination of mitotic and labelling indices

Mitotic index was determined in uterine sections fixed in Carnoy's fluid and stained with iron haematoxylin. All procedures for determination of mitotic and labelling indices were performed under a light microscope. All results were expressed as percentage of dividing or labelled cells.

For determination of mitotic, PCNA- and BrdU-labelling indices for the luminal and glandular epithelia, all cells of the luminal epithelium and the epithelium of the endometrial glands (separately) in whole transverse section were counted, and dividing or PCNA- or BrdU-labelled cells were also counted. Not less than 3000 cells were viewed per rat. Mitotic or labelling indices were then calculated by dividing the number of mitotic or labelled cells into the total number of cells counted.

The mitotic index for endometrial stromal cells was calculated as follows. Whole transverse sections were viewed and all dividing cells were counted. The total number of stromal cells per section was then calculated by counting the number of stromal cells per field of view of

Table 1 Effect of long-term treatment with triamcinolone on body weight, the weight of adrenal glands and thymus of ovariectomized rats. Values are mean $\pm$ S.E.M.

	Treatment	Olive oil (n=5)	Oestradiol (n=7)
Body weight of rat (g) ^a	Saline	221.1 $\pm$ 11.4	217.3 $\pm$ 6.5
	Triamcinolone	158.3 $\pm$ 10.2**	145.7 $\pm$ 7.6***
Adrenal gland weight	Saline	13.7 $\pm$ 0.8	15.1 $\pm$ 0.4
(mg/100 g body weight)	Triamcinolone	9.8 $\pm$ 0.6**	9.9 $\pm$ 0.7***
Thymus weight	Saline	181.1 $\pm$ 24.2	186.4 $\pm$ 30.1
(mg/100 g body weight)	Triamcinolone	128.3 $\pm$ 16.9	132.9 $\pm$ 10.1

Data from rats killed 24 h after oestradiol or olive oil injection are presented. Data obtained 36 and 48 h after oestradiol or olive oil injection did not differ significantly from those presented.

*** $P < 0.001$; ** $P < 0.01$ compared with control animals not treated with triamcinolone (Student's *t*-test).

^aBody weight of animals at the beginning of experiment (day of ovariectomy) was 190 $\pm$ 6.6 g.

the microscope. Then, the area of the microscopic field of view and the area of endometrium in transverse section were found by measuring the dimensions of the field of view and the dimensions of the endometrium respectively using an ocular micrometer. The number of stromal cells per section was then calculated. The mitotic index for stromal cells was calculated by dividing the number of mitoses per section into the total number of stromal cells per section. Not less than 10 000 stromal cells were analysed per rat.

PCNA- and BrdU-labelling indices for endometrial stromal cells were found in the following way. In a section, the number of labelled cells and the total number of stromal cells per field of view were counted in different regions of the endometrium. At least ten fields of view were analysed per section. The labelling index for stromal cells was calculated by dividing the number of labelled cells into the total number of stromal cells per field of view. Not less than 3000 cells were analysed per animal.

Statistical analysis

Arithmetic means and standard errors were calculated for each data group. Significance of differences were evaluated by Student's *t*-test. The significance of the influence of long-term treatment with glucocorticoids at different time points after oestradiol or vehicle injection was determined by two-way ANOVA.

Results

To assess the general glucocorticoid actions of triamcinolone, body weight and the weight of the adrenal glands and thymus were measured. At 24 h after treatment with oestradiol or vehicle along with triamcinolone, body weight and the weight of the adrenal glands and thymus were significantly reduced compared with those of control animals treated with saline (Table 1). The data obtained at 36 and 48 h after injection of oestradiol or vehicle along

with triamcinolone were also diminished and did not differ from those obtained at the 24 h point.

Treatment with oestradiol and saline caused a large increase in the mitotic indices and BrdU- and PCNA-labelling indices of all the uterine structures compared with those of rats not treated with oestradiol. In all the tissues, the values of all parameters continued to increase from 24 to 48 h after oestradiol injection (Fig. 1).

In the luminal epithelium of the endometrium from animals treated with oestradiol and triamcinolone, the mitotic index and BrdU- and PCNA-labelling indices were decreased at 24 h (Figs 1 and 2) and 48 h, and increased at 36 h after oestradiol injection compared with those of rats treated with oestradiol and saline (Fig. 1). Mitotic index and PCNA-labelling index for the glandular epithelium were similar at 24 h (slightly decreased), increased at 36 h and decreased at 48 h compared with control animals treated with oestradiol and saline. BrdU-labelling index for glandular epithelium was higher at 24 h and lower at 36 and 48 h than those of control rats (Fig. 1). All differences in the luminal epithelium were statistically significant ($P < 0.05$), but those in the glandular epithelium were not so large, especially at 24 and 36 h, and were only statistically significant at 36 h for the mitotic index and at 48 h for the BrdU and PCNA-labelling indices (Fig. 1).

Treatment with triamcinolone along with oestradiol led to very interesting changes in the stromal cells, all the parameters of which at all periods were increased compared with control rats treated with oestradiol and saline (Fig. 1). Most changes were observed at the 24 h time point (Fig. 2). At this point, all differences in mitotic index and BrdU- and PCNA-labelling indices were statistically significant. Results of two-way ANOVA indicate that the influence of long-term treatment with glucocorticoid on proliferative parameters in oestradiol-treated animals is significant only for stromal cells (Table 2).

At 24 h after treatment with olive oil and saline, mitotic indices and BrdU- and PCNA-labelling indices in all the structures were very low compared with those of animals treated with oestradiol (Fig. 1). The data obtained at 36 and 48 h after treatment with olive oil and saline did not

Figure 1 Effect of long-term treatment with the glucocorticoid triamcinolone on the oestradiol-induced increase in mitotic index, BrdU-labelling index and PCNA-labelling index in the compartments of ovariectomized rat uterus. Data for rats treated with oestradiol and triamcinolone (darkly shaded bars) compared with those of control rats treated with oestradiol and saline (lightly shaded bars) are presented. Crystal triamcinolone acetate (single dose 0.1 mg/100 g, i.m.) was injected once a week for 29 days. On the second day after the last triamcinolone administration, the rats were treated with a single injection of oestradiol dipropionate (5 µg/100 g, s.c.) or vehicle (olive oil). At 2 h before the rats were killed, BrdU (2 mg/100 g i.p.) was given. Measurements were made at 24, 36 and 48 h after the oestradiol injection. The data at 0 h are those for rats treated with olive oil and saline 24 h after the oil injection. No changes were observed in the data of any of the groups not treated with oestradiol. There were seven rats per oestradiol-treated group, and five animals per group not treated with oestradiol. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with the data for rats treated with saline and oestradiol (Student's *t*-test). Values are means $\pm$ S.E.

differ significantly from those obtained at 24 h. In animals treated with olive oil, triamcinolone did not produce any significant changes in any of the parameters tested at any of the time points, compared with those of rats treated with saline. Using two-way ANOVA, no significant effect of triamcinolone was observed in the groups not treated with oestradiol.

In conclusion, long-term treatment with glucocorticoid results in dissimilar changes in oestradiol-induced proliferation of epithelial and connective-tissue (stroma) components of the uterus. In luminal and glandular epithelia, an initial reduction in proliferation at 24 h is changed to an increase at 36 h and then a further reduction at 48 h after oestradiol injection. In stromal cells of the endometrium,

Figure 2 Photomicrographs demonstrating BrdU- (A-1, A-2) and PCNA- (B-1, B-2) labelled cells in the luminal epithelium (L), glandular epithelium (G; white letters) and stromal cells (S) of the endometrium of ovariectomized rats treated with oestradiol and triamcinolone (A-2, B-2) or saline (A-1, B-1), at 24 h after the oestradiol injection. No staining was observed in the negative control in which normal goat serum was substituted for primary antibodies. Scale bar 20 μ m.

chronic triamcinolone treatment leads to a large constant increase in oestradiol-induced proliferation throughout the experiment. The treatment protocol has no effect on the parameters in the absence of oestradiol administration.

Discussion

Long-term glucocorticoid treatment was used in these experiments. The effectiveness of the treatment protocol was controlled by analysing well-known general actions of glucocorticoid, such as decreasing body weight and the weight of the thymus and adrenal glands. These par-

ameters were measured. Body weight and the weight of the thymus and adrenal glands were significantly reduced after the treatment with triamcinolone. Triamcinolone acetone was used in crystal form, which produces an increased level of the hormone for more than a week (Mikhail *et al.* 1977, Lypko *et al.* 1983). Therefore it can be concluded that the treatment schedule used was appropriate to induce a general chronic hypercortico-steroid effect.

The extent of oestradiol-induced proliferation in the uterus was analysed using three criteria: mitotic index and BrdU- and PCNA-labelling indices. The mitotic index shows how many dividing cells are present in a tissue and

Table 2 Two-way ANOVA demonstrating the significance of the influence of long-term treatment with triamcinolone on oestradiol-induced effects in the uterus of ovariectomized rats at different time points after oestradiol injection

	Mitotic index	BrdU-labelling index	PCNA-labelling index
Uterine structure			
Luminal epithelium	NS	NS	NS
Glandular epithelium	NS	$P<0.05$	NS
Stromal cells	$P<0.01$	$P<0.001$	$P<0.001$

NS, Not significant.

gives information about the M-phase of the cell cycle. BrdU is incorporated into DNA during the S-phase. Therefore, if BrdU is injected 2 h before the animals are killed, the BrdU-labelling index shows the number of cells that are in or have just passed through the S-phase. PCNA is an auxiliary protein of DNA polymerase δ (36 kDa) which is synthesized in late G_1 - and S-phases of the cell cycle and is required for DNA synthesis and repair (Woods *et al.* 1991, Sawtell *et al.* 1995). PCNA has a half-life of 12 h and is therefore present in cells that are in the G_1 -, S-, G_2 - and M-phases of the cell cycle (Woods *et al.* 1991, Sawtell *et al.* 1995). Thus these three parameters are appropriate for estimating not only the general proliferative activity of a tissue, but also any shifts in the phases of the cell cycle, which are strongly affected by oestrogen hormones. Oestrogens lead to a reduction in the duration of all the stages of the cell cycle and drive cells from the G_0 - to the G_1 -phase; this is followed by an increase in the number of cells in and passing through the G_1 - and S-phases, as well as the quantity of dividing cells (Quarmby & Korach 1984, Evans *et al.* 1990, Galand & de Maertelaer 1992, Rumpel *et al.* 1995). Therefore the increase in the mitotic activity and the number of BrdU- and PCNA-positive cells in all uterine cell types after oestradiol treatment, observed in the present study, is a direct reflection of these processes.

Treatment with oestradiol and triamcinolone produced an increase in all proliferative parameters at all time points in stromal cells of the endometrium. This indicates that the number of cycling cells in the stroma is increased. In luminal and glandular epithelia, the effect of long-term treatment with triamcinolone is different. The treatment leads to an initial reduction in proliferation at 24 h, followed by a significant increase at 36 h and a further reduction at 48 h after oestradiol injection. The decrease in not only the mitotic and BrdU-labelling indices but also the PCNA-labelling index at 24 h is probably associated with an arrest of the cells in early G_1 -phase, because PCNA begins to be synthesized from the late G_1 -period (Woods *et al.* 1991, Sawtell *et al.* 1995). As a result, more cells are accumulated in the early G_1 -phase, which are ready to go on. These cells then begin to drive into the late G_1 -phase and further through the cell cycle, and at

36 h, this situation is reflected by an increase in the number of PCNA- and BrdU-positive cells as well as the mitotic index. However, at 48 h, all the parameters were again decreased. This is probably associated with these cells being arrested in the next G_1 -phase or with blocking of the entry of these cells into the next cell cycle. Hence, it may be suggested that long-term treatment with triamcinolone leads to an increase in oestradiol-induced proliferative reactions in endometrial stroma and to a decrease in the same in luminal and glandular epithelia, and a key point affected by triamcinolone in epithelial cells is the early G_1 -phase of the cell cycle. It is also shown that no effect of long-term treatment with triamcinolone was observed in the absence of oestradiol. Therefore, it may be proposed that triamcinolone affects the mechanism of oestrogen action on the uterus.

The effect of triamcinolone was probably mediated by glucocorticoid receptors, the presence of which in uterine tissues is well documented (Panko *et al.* 1981, Giannopoulos *et al.* 1983). This pathway was confirmed to be the one by which effects of acute treatment with glucocorticoids occur (Campbell 1978, Atkinson & Adams 1988, Rabin *et al.* 1990). Acute treatment with glucocorticoids has been shown to greatly diminish oestrogen-stimulated proliferative activity as well as the number of oestrogen receptors in all the uterine cell types (Atkinson & Adams 1988, Rabin *et al.* 1990, Bigsby 1993). The present work also demonstrates a decrease in proliferation in the luminal and glandular epithelia, where the number of glucocorticoid receptors may also be reduced. However, the effect of long-term treatment with glucocorticoids in stromal cells of the endometrium is different from the effect of short-term treatment. It is possible that the mechanisms involved in the chronic effect of glucocorticoids on stromal cells are also different. Treatment with glucocorticoids for a long time probably leads to a decrease in the number of glucocorticoid receptors in stromal cells. This phenomenon of down-regulation for glucocorticoid receptors has been shown in different target tissues after long-term treatment with glucocorticoids (Schmidt & Meyer 1994, Herman *et al.* 1995, Sapolsky 1996). Interestingly, prolonged administration of oestrogen steroids also leads to a decrease in the number of oestrogen

receptors in the uterus (Mukku *et al.* 1982, Medlock *et al.* 1991).

It cannot be excluded that progesterone receptors are involved in the action of triamcinolone. Progesterone has been documented to stimulate oestradiol-dependent proliferation of stromal cells (Martin & Finn 1969, Finn *et al.* 1995) and to diminish this process in uterine epithelium, where it affects the G₁-phase of the cell cycle and blocks the entry of epithelial cells into a second round of DNA synthesis (Das & Martin 1973, Martin *et al.* 1973). The present results are similar, and it is possible that the effect of triamcinolone on the uterus is achieved through progesterone receptors. Moreover, cross-reactivity between glucocorticoid and progesterone receptors has been documented (Panko *et al.* 1981, Von der Ahe *et al.* 1985).

Another possible pathway may involve prolactin. It is known that long-term treatment with glucocorticoids can lead to a decrease in prolactin concentration (Piroli *et al.* 1993, Taylor *et al.* 1995), and prolactin has been shown to reduce oestradiol-induced proliferation in the uterus (Gunin 1996). Therefore the effect of triamcinolone, at least on stromal cells, may be mediated through a decrease in the level of prolactin.

So, the mechanisms involved in the observed effect remain unclear, and further experiments are needed to define it. In conclusion, it should be noted that this is the first report that demonstrates not only the effect of long-term glucocorticoid action on the uterine response to oestradiol but also a large increase in oestrogen-induced proliferation in the uterus after the administration of glucocorticoids. We hope that these data will lead to a deeper understanding of the origin and development of oestrogen-dependent cancer of the female reproductive system.

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