PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 68 (1999) 203–209

The Journal of Steroid Biochemistry & Molecular Biology

Regulation of thioredoxin mRNA in the rat uterus by gonadal steroids

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Received 14 October 1998; accepted 12 January 1999

Abstract

Estradiol has been shown to increase the level of thioredoxin mRNA in the uterus of the ovariectomized (ovx) rat. In this study the influence of progesterone, androgens, the anti-estrogen ICI 182780 and the anti-androgen Flutamid on thioredoxin expression, has been studied in the rat uterus. Thioredoxin mRNA concentrations were determined by solution hybridization. Ovx rats treated with progesterone alone showed no effect on thioredoxin expression. Combined treatment of ICI 182780 and estradiol attenuated the estradiol-induced increase in thioredoxin mRNA. When ovx rats were treated with a testosterone depot, the amount of thioredoxin mRNA was increased five-fold after 48 h and remained at that level during the rest of the 168 h monitored. A similar increase in thioredoxin mRNA could be seen after 5α-dihydrotestosterone treatment, indicating a true androgenic effect. In addition, the anti-androgen Flutamid attenuated the thioredoxin mRNA increase seen after 5α-dihydrotestosterone treatment alone.

It is concluded that thioredoxin mRNA is regulated by growth promoting gonadal steroids in the rat uterus. The attenuation of the estrogen and androgen-induced increases of the thioredoxin mRNA with ICI 182780 and Flutamid, indicate that the effect is mediated via the estrogen receptor and androgen receptor respectively. None of these hormones affected the hepatic thioredoxin mRNA level in the same animals.

1. Introduction

Thioredoxin, with two redox active vicinal cysteine residues, is a small multifunctional protein, which classically functions as a dithiol hydrogen donor for the enzyme ribonucleotide reductase, which is essential for DNA synthesis [1]. In addition, thioredoxin participates in the redox regulation of the activity of different enzymes, receptors and transcription factors via dithiol/disulfide interchange reactions [2–8]. Thioredoxin may also be secreted by activated lymphocytes or by normal liver cells, where it may act as a growth factor [9–11] or operate in synergy with cytokines to stimulate cell growth [6,12,13]. We have previously shown that the thioredoxin mRNA level in the uterus is increased after E_2 treatment of ovx rats [14] and that the thioredoxin mRNA level in cervix correlates with the serum estradiol level in non-pregnant women [15].

The expression of human thioredoxin (isolated as adult T-cell leukemia derived factor, ADF) has been found very closely associated with the activity of steroid-producing cells in the human [16] and rat [17] ovary. In the human fetus, steroid-producing cells in the adrenal cortex, the ovary and the testis have been shown to present strong immunohistochemical reactivity for thioredoxin [18].

In a recent paper, thioredoxin expression in the human endometrium was shown to be highest in the early secretory phase of the menstrual cycle [19]. This period is influenced by the estrogen peak prior to ovulation and shows increasing levels of progesterone. It is difficult to distinguish between estrogen and progesterone influence in human tissue samples, since both hor-

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0960-0760/99/$ - see front matter
PII: S0960-0760(99)00031-X

Table 1
Outline of experiment 2. There were six rats in each group except for group 3, where one rat was found to be incompletely ovariectomized and excluded from the study. The rats were treated s.c. in the neck with 2.5 μg E_2, 1 mg P or vehicle (propyleneglycol)

Group		Treatment		
		Day 1	Day 2	Day 3
1	Ovariectomized control (ovxc)	Vehicle	Vehicle	Sacrificed
2	24 h/E24 h	2.5 μg E_2	Sacrificed after 24 h	
3	24 h/P24 h	1 mg P	Sacrificed after 24 h	
4	48 h/E24 h + E24 h	2.5 μg E_2	2.5 μg E_2	Sacrificed after 48 h
5	48 h/P24 h + P24 h	1 mg P	1 mg P	Sacrificed after 48 h
6	48 h/E24 h + P24 h	2.5 μg E_2	1 mg P	Sacrificed after 48 h
7	48 h/P24 h + E24 h	1 mg P	2.5 μg E_2	Sacrificed after 48 h

mones are present. Therefore we have studied the effects of hormone treatment in the uterus in a rat model-system, since ovariectomy can be used as a tool to limit endogenous hormone exposure. In the present study the regulation of the thioredoxin expression in the rat uterus, during the estrous cycle and after estradiol (E_2), progesterone (P), testosterone (T), 5α-dihydrotestosterone (5αdhT), the anti-estrogen ICI 182780 (ICI) and/or the anti-androgen Flutamid® (F) treatment, was examined, and its possible role in the process of uterine growth discussed.

2. Material and methods

Adult 55–60 days old female Sprague-Dawley rats weighing approx. 250 g, were used. The animals were housed in a controlled environment at 20°C on an illumination schedule of 12 h light/12 h dark each day. Standard pellet food and water were provided ad libitum. Ovariectomy was performed dorsally under light ether anesthesia 14 days before hormone treatment. The hypophysectomized rats were purchased from Møllegaards Breeding Center Ltd (Skensved, Denmark). The animal studies were approved by the Committee on Animal Care in Sweden.

2.1. Hormones

17β-E_2, P and 5αdhT were purchased from Sigma Co. (St Louis, Missouri), and dissolved in 99.5% ethanol at a high concentration and then diluted with propyleneglycol to the proper concentration. The final ethanol concentration in the injections/infusions was less than 5%. For the time-course study Testoviron-Depot® (135 mg/ml) (Schering AG, Berlin, Germany) was used. The antiestrogen ICI 182780 [20] was a generous gift from Dr A.E. Wakeling, Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK, and the Flutamid® was kindly supplied by Schering Cooperation, Bloomfield, NJ.

2.2. Experiment 1

Forty-seven normal cycling rats were used and vaginal smears were taken each day to determine the stage in the estrous cycle. When the rats showed regular cycles of 4–5 days duration, 11–13 animals were sacrificed in each of the four different stages during the following cycle. The vaginal smearing was performed as described in Experimental Endocrinology [21].

2.3. Experiment 2

Forty-one rats were ovx and 2 weeks later subjected to hormone treatment. The animals were injected subcutaneously in the neck with the respective hormone in a volume of 100 μl with propyleneglycol as vehicle 08:00 h, day 1 and, when applicable, day 2. The rats were sacrificed 08:00 h, day 2 or 3. 17β-estradiol (2.5 μg) and/or progesterone (1 mg) were given as shown in Table 1.

2.4. Experiment 3

Twenty-two ovx animals were treated with E_2 (2.5 μg/rat), ICI 182780 (100 μg/rat) or a combination of these, 24 h before sacrifice. The ovx control group received vehicle only (propyleneglycol).

2.5. Experiment 4

A time-course study of T treatment was carried out in 42 ovx rats. The hormone was administered as a depot. The animals were given an i.m. injection of 20 mg in 150 μl ol. ricini at time 0, and six rats each time-point were then sacrificed after 6, 24, 48, 72, 96 and 168 h.

2.6. Experiment 5

Twenty-four hypophysectomized (hx) rats were treated with E_2 by constant infusion via osmotic mini-

pumps at 4 μg/h, and T and 5αdhT at 20 μg/h, for 7 days before sacrifice. The hormones were administered in Alzet osmotic pumps (Alza, Palo Alto, California). The pumps were placed subcutaneously in the neck under light ether anesthesia.

2.7. Experiment 6

Eighteen ovx rats were treated with a single injection of either 5αdhT (500 μg), F (5 mg) or a combination of both. They were sacrificed 24 h after the injections.

2.8. Preparation of total nucleic acids

Total nucleic acids (TNA) were prepared as described before [22]. In short, the tissues were homogenized and digested with proteinase K in an SDS-containing buffer, followed by subsequent extraction with phenol-chloroform as described by Durnam and Palmiter [23]. The concentration of total DNA in the TNA samples was measured fluorometrically at the wavelength 458 nm with Hoechst Dye 33258 [24].

2.9. Hybridization probe

The probe used for the thioredoxin mRNA determinations was derived from a clone of human thioredoxin cDNA as described previously [25]. The thioredoxin cDNA (open reading frame coding for 105 amino acid residues) [26] subcloned into plasmid pACA [27] was isolated by EcoR1 digestion and subsequently subcloned into the EcoR1 site of the pGEM3Z vector. Two subclones with opposite orientations were selected and digested with SmaI. Sense and anti-sense RNA were obtained using T7 RNA polymerase. For measurements of specific mRNA, probes were synthesized in vitro and radiolabeled with ^{35}S-UTP (Amersham, UK). The in vitro synthesis of radioactive cRNA was performed essentially as described by Melton et al. 1984 [28] using reagents supplied from Promega Biotech (Madison, WI). A Northern blot showing that the human probe cross-reacts with the thioredoxin mRNA in rat tissues, resulting in a band of the expected size (approx. 0.6 kb), was published in Sahlin et al. (1997) [14].

2.10. Solution hybridization analysis of thioredoxin mRNA

This method was performed as described before [29], with a modification in order to be quantitative as presented in Sahlin et al. (1997) [14]. The modification entailed that the radioactivity on the filters, recovered following the collection of the precipitate of the labeled hybrids protected from RNase digestion, were compared with a standard curve of known amounts of in vitro synthesized mRNA complementary to the probe used. Results are expressed as amol (10^{-18}) mRNA/μg DNA in the TNA sample.

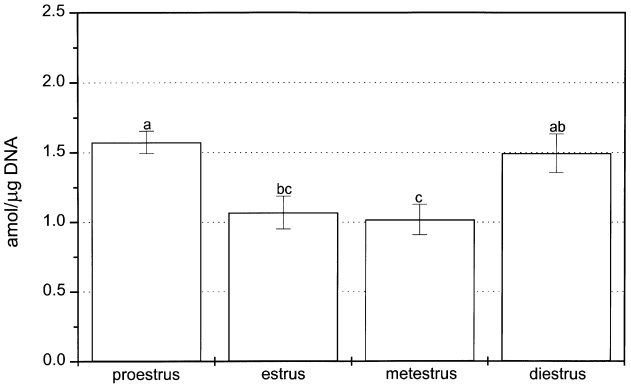

Fig. 1. The levels of thioredoxin mRNA in uteri from rats during their estrous cycle. There were 11 rats in the proestrus and diestrus groups, 13 rats in the estrus group and 12 animals in the metestrus group. Values with different letter designations are significantly different ($P < 0.05$).

2.11. Statistics

The results are presented as mean ± S.E.M. The evaluation was made by ANOVA and significance ($P < 0.05$) determined by Scheffés test [30]. Values with the same letter designation are not significantly different ($P > 0.05$).

3. Results

3.1. Normal cycling rats

The uterine thioredoxin mRNA level was higher

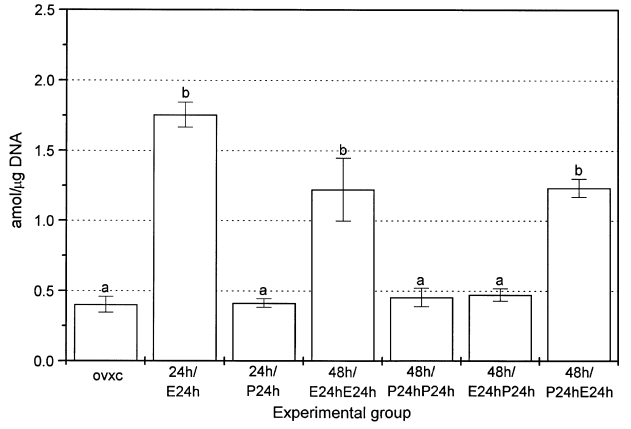

Fig. 2. The levels of thioredoxin mRNA in uteri from ovx rats treated with E_2, P or combinations of these. Ovxc is the ovx control group. There were six rats in all groups except the 24 h/P24 h, where it was five animals. Values with different letter designations are significantly different ($P < 0.05$).

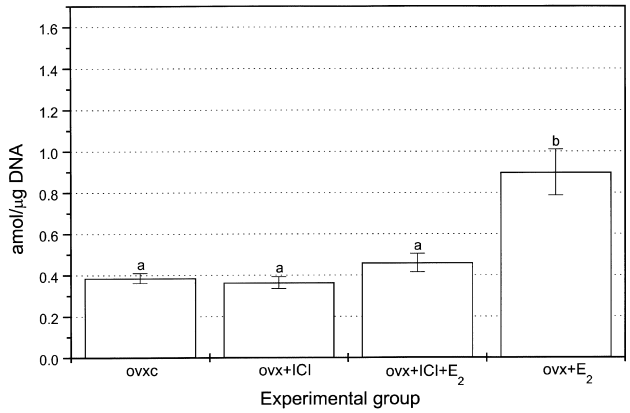

Fig. 3. The levels of thioredoxin mRNA in uteri from ovx rats treated with estradiol and/or the antiestrogen ICI 182780 as single injections and sacrificed 24 h later. Ovxc is the ovx control group. $N=5$ in the ovxc and ovxc + ICI groups, $n=6$ in the ovx + ICI + E_2 and ovx + E_2 groups. Values with different letter designations are significantly different ($P < 0.05$).

during the proestrus phase as compared to estrus and metestrus. The level in the diestrus group was higher than in the metestrus group (Fig. 1).

3.2. Ovariectomized rats treated with estradiol and/or progesterone

When ovx rats were treated with E_2 or P or combinations of these, only the animals receiving E_2 the last 24 h of treatment, had increased levels of thioredoxin mRNA (Fig. 2). P had no effect on the thioredoxin mRNA level on its own (Fig. 2). In the livers of these animals the basal thioredoxin mRNA level was approximately ten-fold higher than in the uteri, but no variation between the treatment groups were found (data not shown).

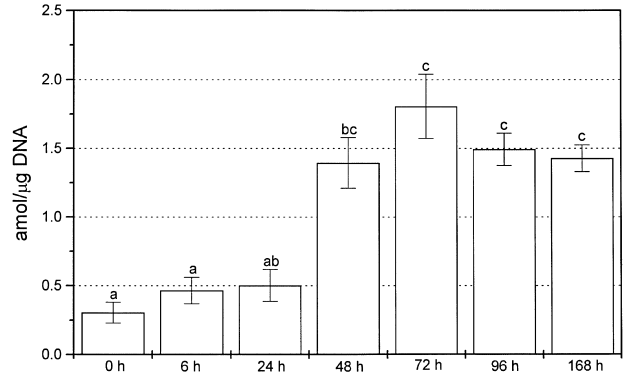

Fig. 4. The levels of thioredoxin mRNA in uteri from ovx rats treated with a depot of T for up to one week. $N=6$ in each group. The group 0 h is the ovx control group prior to treatment. Values with different letter designations are significantly different ($P < 0.05$).

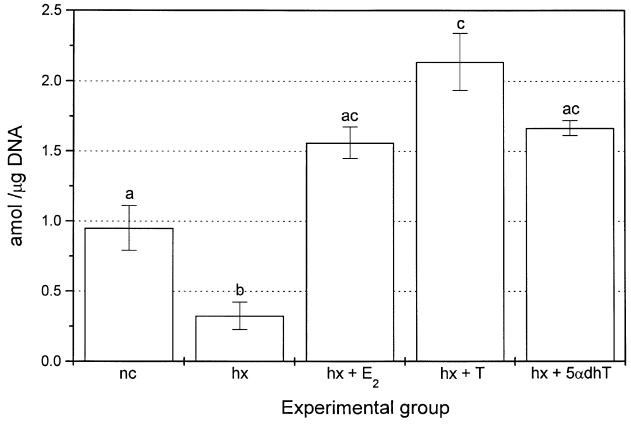

Fig. 5. The levels of thioredoxin mRNA in uteri from hx rats after treatment with E_2, T or 5αdhT for 7 days. $N=6$ in the nc (=normal control) and hxc (=hypophysectomized control) groups, $n=5$ in the hormone treated groups. Values with different letter designations are significantly different ($P < 0.05$).

3.3. Ovariectomized rats treated with estradiol and/or the antiestrogen ICI 182780

ICI 182780 had no effect on the thioredoxin mRNA level on its own (Fig. 3). Administered together with estradiol the significant increase in the thioredoxin mRNA level that is seen after estradiol treatment was attenuated (Fig. 3).

3.4. Time-course study of testosterone treatment in ovx rats

In the animals treated with the T depot, the thioredoxin mRNA level was significantly increased after 48 h, compared to time 0, and remained increased during the rest of the week monitored (Fig. 4). In the livers of these animals no differences in the thioredoxin

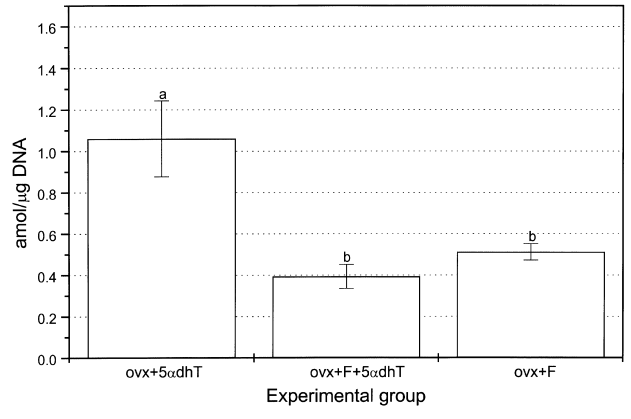

Fig. 6. The levels of thioredoxin mRNA in the uteri of ovx rats 24 h after an injection with either the androgen 5αdhT, the anti-androgen F or a combination of both. $N=6$ in each group. Values with different letter designations are significantly different ($P < 0.05$).

mRNA levels between the time-points were seen (data not shown).

3.5. Hypophysectomized rats treated with E_2, T or 5αdhT for one week

The androgen 5αdhT increased the thioredoxin mRNA level in a similar way as T (Fig. 5), i.e. around five-fold, after 7 days of treatment. The high dose E_2 treatment also increased the thioredoxin mRNA five-fold after 7 days.

3.6. Ovx rats treated with 5αdhT and/or the androgen receptor blocker Flutamid

When the anti-androgen F was given alone or in combination with 5αdhT, the thioredoxin mRNA level was decreased compared to the level seen after 5αdhT treatment alone (Fig. 6).

4. Discussion

It has previously been shown that E_2 treatment of ovx rats induced a tissue specific increase in the uterine thioredoxin mRNA level [14]. The E_2 concentration during the estrous cycle in the rat was highest in the proestrus phase [22], which is in accordance with the increased thioredoxin mRNA level in the proestrus group seen in this study. The uterine wet weight was also highest in proestrus, and estrus, whereas the weights in metestrus and diestrus were significantly decreased compared to the proestrus group [22]. Thus, the weights co-varied with the high plasma E_2 and low plasma P levels. In this study we found that the thioredoxin mRNA level in the diestrus group is also incresed in the same range as during proestrus, which implicates that the estradiol level is not the only regulator of thioredoxin expression in the uterus.

In a recent paper Maruyama et al. have shown that the thioredoxin mRNA level in the human endometrium is three-fold higher in the early secretory phase as compared to the other phases during the menstrual cycle [19]. In the early secretory phase the endometrium is prepared for implantation of the fertilized egg, by estrogen influence and during exposure to increasing levels of progesterone. In this study the rats that were treated with E_2 or P or combinations of these, showed no effect of P on the level of thioredoxin mRNA in the uterus. Still we cannot exclude that P could influence the susceptibility to E_2 stimulation, although without influence on its own on the thioredoxin mRNA level. Whether P is involved in the induction of thioredoxin in the human uterus is not known. The finding that the cervical thioredoxin mRNA level is correlated to the serum E_2, but not P, concentration, in non-pregnant women [15] suggests that P by itself is without effect and that the data from the rat study apply also to the human uterus.

Treatment with the antiestrogen ICI 182780 attenuated the estradiol-induced increase in the thioredoxin mRNA level, indicating that the effect is mediated via the estrogen receptor (ER). ICI 182870 is acting as a pure antiestrogen, inhibiting E_2-dependent activation, of both ERα and ERβ [31]. In the rat uterus there is more ERα than ERβ [32]. We cannot determine from these data, however, via which of the two receptors the thioredoxin mRNA level is regulated or if there is a combined effect of the receptors.

Testosterone was also shown to increase the uterine thioredoxin mRNA level significantly 48 h after initiation of hormone treatment and maintained this increase at least during the rest of the experimental period of 7 days. To exclude the possibility that the effect of T was due to aromatization into E_2, also the effect of the non-aromatizable androgen 5αdhT was tested. To test if the effect of androgens and E_2 was dependent on any pituitary derived factor, an experiment was carried out in hypophysectomized rats. In the uteri of hx rats 5αdhT increased the thioredoxin mRNA five-fold after 7 days of treatment, quite similar to treatment with T or E_2. In ovx rats the level of thioredoxin mRNA seen after 5αdhT treatment was decreased by a concomitant injection of the anti-androgen Flutamid®. The experiments with 5αdhT show that the effect on thioredoxin expression after androgen treatment is a true androgenic phenomenon and mediated via the androgen receptor. We also found that E_2, T and 5αdhT have a similar effect on the uterine thioredoxin mRNA level in hx as in ovx rats.

The present findings imply that thioredoxin expression is increased in uterine tissue when growth is induced, since both E_2 and androgens, but not P, increase uterine growth on its own. This could be due to the increased need of thioredoxin in DNA synthesis [2], ER activation [7], transcription factor regulation [3], growth factor or co-cytokine action [4,5], or combinations of these.

Uterine IGF-I expression is also increased by estrogens [22,33] and androgens [34]. A common finding in the thioredoxin and IGF-I genes are AP-1 binding motifs in their promoter regions [35,36]. No classical estrogen responsive element (ERE) has been found in the promoter of the IGF-I gene, but still estrogen regulates its expression [36]. The AP-1 binding motif in the promoter has been shown to be essential for estrogen regulation of the chicken IGF-I gene [36]. We found one half (AGGTCA) of the palindromic consensus sequence for ERE (AGGTCAnnnTGACCT) in the promoter region of thioredoxin (−717 to −712) as published by Kaghad et al. [35]. For estrogen regu-

lation of the IGF-I gene it was found that the DNA binding domain of the ER was essential for activation of the AP-1 site [36]. Umayahara et al. concluded that a post-transcriptional modulation of AP-1 activity by estrogen might be a widely utilized signaling pathway for regulation of genes that lack conventional ERE [36]. The ER is then activating the genes via the AP-1 site by interaction with the Fos/Jun complex. Furthermore, Paech et al. has shown in a recent paper that the AP-1 binding site is only activated by 17β-E_2 via ERα, whereas 17β–E_2 via ERβ inhibited transcription [37]. These data, combined with the anti-estrogen results discussed above, make us suggest that the increase in thioredoxin mRNA after 17β-E_2 treatment is mediated via ERα.

In summary the growth promoting gonadal steroids, i.e. estrogens and androgens, increase the expression of thioredoxin in the rat uterus, whereas progesterone does not affect the expression. The effect is tissue specific.

Acknowledgements

We thank Monica Lindberg for excellent technical assistance. This work was supported by grants from the Swedish Medical Research Council (grants 03X-3972 and 13X-3529), Magn. Bergvalls stiftelse, The Swedish Society of Medicine and Karolinska Institutets Fonder.

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