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WEAK ESTROGENIC ACTIVITY OF PHENOL RED IN THE PITUITARY GONADOTROPH: RE-EVALUATION OF ESTROGEN AND ANTIESTROGEN EFFECTS

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Summary—Phenol Red (Phr) which is widely used as a pH indicator in cell culture media has recently been described to possess estrogenic activity in different cell types. In the present study we investigated if the dye shows such activity on LH secretion of cultivated rat pituitary cells and controlled the established effects of estradiol (E_2) and keoxifene (K) in this model in the absence of Phenol Red. 24 h treatment of pituitary cell cultures with Phr led to enhancement of GnRH-stimulated LH secretion whereas 4 h treatment reduced LH secretion. When the cells received E_2 instead of Phr for the indicated incubation periods we observed nearly identical results i.e. a short-term inhibitory and a long-term stimulatory effect on LH secretion. 24 h treatment of pituitary cell cultures with increasing concentrations of Phr led to a stimulatory effect on GnRH-stimulated LH secretion an effect that occurred at 10 μM got maximal at 100 μM and was lost at higher concentrations resulting in a bell-shaped dose–response curve. The inhibitory action of Phr was present at concentrations $\geqslant 10\,\mu$M. Both effects could be blocked by the antiestrogen K indicating their specificity. K has recently been described to induce an antigonadotrophic effect in this model. Although high concentrations of the antiestrogen were still able to inhibit LH secretion this effect was not present at lower concentrations when Phr-free culture medium was used in the experiments. Thus Phr showed weak estrogenic activity in the gonodotroph. The established actions of E_2 and K on LH secretion were qualitatively reproducible when Phr was excluded from the culture medium.

INTRODUCTION

Phenol Red which is widely used as a pH indicator in cell and tissue culture media has recently been described to possess weak estrogenic activity in MCF-7 breast cancer cells [1]. This finding has been confirmed in a number of different cell types and binding of the dye to the estrogen receptor of uterine tissue and MCF-7 cells has been demonstrated [1–3]. Therefore the results and implications of those studies in which estrogen-responsive cells or tissues cultured in the presence of Phenol Red had been used needed to be reevaluated [4–8]. Actions of estrogens and antiestrogens on gonadotrophin secretion have been studied extensively in the model of cultured rat anterior pituitary cells. Estradiol is capable of inducing an inhibitory effect on GnRH-induced LH secretion after short-term and a stimulatory effect after long-term incubation of such cells with the steroid [9–11]. Antiestrogens also cause negative and positive effects on gonadotrophin secretion depending on the duration of treatment, the concentration, and the type of antiestrogen that was used [12–14].

In the present study we investigated the effects of two different Phenol Red preparations on LH secretion of rat pituitary cells in static culture and compared them to the actions of estradiol in this model. We also verified the established estradiol effects in Phenol Red-free culture medium. In a recent study we have shown that keoxifene has an inhibitory effect on LH secretion of cultured rat pituitary cells [15]. Our previous data were controlled by repeating the experiments in the absence of Phr in order to determine if the observed negative effect of keoxifene is due to the antagonism of the estrogenic action of Phenol Red or has to be considered as an effect that is caused by the antiestrogen *per se*.

EXPERIMENTAL

Hormones

Estradiol was obtained from Schering (purity > 99.9%, Berlin, Germany). Keoxifene (LY 156 758) was a gift from Lilly Research Laboratories (Eli Lilly and Co., Indianapolis, Ind.). GnRH was purchased from Sigma (Deisenhofen, Germany). Keoxifene was dissolved in dimethylsulfoxide (DMSO), estradiol

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was prepared in ethanol and GnRH in PBS containing 1 mg/ml BSA.

Chemicals

The Phenol Red preparations were either purchased from Biochrom (Berlin, Germany, lot number 5 S 18) or from Sigma (lot number 55 F-34126).

Pituitary cell preparation and culture conditions

Anterior pituitary glands obtained from adult female Wistar rats (180–200 g) at random stages of the estrous cycle (Winkelman, Borchen-Kirchborchen, Germany) were used for the preparation of primary cultures of pituitary cells as previously described [16]. The cells were cultured in medium 199 without Phenol Red with Hank's salts and L-glutamine (Biochrom, Berlin, Germany) supplemented with 1.4 mg/ml sodium bicarbonate, 10 μg/ml streptomycin, 100 U/ml penicillin, and 10% horse serum (Biochrom, Berlin, Germany) pretreated with 2% charcoal (Norit A) and 0.2% Dextran T 70 (Pharmacia, Uppsala, Sweden) (incubation medium) on multiwell culture dishes at a density of 2.5×10^5 cells per well.

The cultures were maintained in a water-saturated atmosphere of 95% air–5% CO_2 at 37°C. They were used for experiments between days 3 and 5 of culture. Before hormonal, antihormonal and Phenol Red treatment the cultures were washed with freshly prepared medium. Prolonged-term incubations (>4 h) were performed in incubation medium (see above) and short-term treatments (<4 h) in the same medium containing 0.1% BSA instead of 10% horse serum. Estradiol and keoxifene were added to the required medium from appropriate stock solutions in ethanol or DMSO. Respective control cultures were exposed to the medium containing the same quantity of ethanol and/or DMSO without hormones (vehicle). The final concentration of ethanol or DMSO was 0.1%. Phenol Red (Biochrom or Sigma) was directly dissolved in the culture medium.

Before the cells were stimulated with GnRH they were washed with serum-free medium and incubated for 1 h; then the medium was renewed again and GnRH was directly added to the cultures in 20 μl volumes; controls received 20 μl PBS containing 0.1% BSA. The media were removed for LH assay after a 3 h simulation period. The cells were checked for viability by the trypan blue exclusion method after each experiment. None of the performed treatments had toxic effects. All experiments were performed in triplicate and were repeated at least twice. The data were derived from experiments using Phenol Red obtained from Sigma or Biochrom. The results of experiments with Phenol Red from Sigma and Biochrom were quantitatively and qualitatively indistinguishable.

Short-term and long-term actions of Phenol Red and estradiol on LH secretion

Pituitary cell cultures were incubated in Phenol Red-free medium and treated for 4 or 24 h with 1 nM estradiol. Another group of cell cultures was incubated for 4 or 24 h with medium 199 containing Phenol Red (50 μM). Control cultures received vehicle. During the last 3 h of the incubation periods the cells were stimulated with increasing concentrations of GnRH (10 pM–1 μM).

Effects of increasing concentrations of Phenol Red on LH secretion

To evaluate the dose–response characteristics of Phenol Red pituitary cell cultures were incubated with increasing concentrations of the dye (100 pM–1 mM) for 4 or 24 h and stimulated with a submaximal dose of GnRH (1 nM) during the last 3 h of the incubation periods.

Effects of keoxifene on long-term and short-term actions of Phenol Red and estradiol on LH secretion

To determine the specificity of the Phenol Red actions pituitary cell cultures were incubated in medium 199 supplemented with 100 μM Phenol Red with or without 100 nM keoxifene for 4 or 24 h. Another group of cell cultures cultivated in Phenol Red-free medium 199 received 1 nM estradiol instead of Phenol Red. The cells were stimulated with 1 nM GnRH during the last 3 h of the incubation periods.

Effects of increasing concentrations of keoxifene on LH secretion of pituitary cells cultivated in medium 199 with or without Phenol Red

To evaluate possible interactions between Phenol Red and the antiestrogen keoxifene pituitary cells cultivated in medium 199 with or without Phenol Red (50 μM) were incubated with increasing concentrations of keoxifene (1 pM–10 μM) for 24 h and stimulated with 1 nM GnRH during the last 3 h of the incubation period.

RIA and data analysis

LH content of the culture media was determined by RIA using the reference preparation RP-2 (AFP-5666 C) provided by the National Pituitary Agency Baltimore, Md [17]. The LH release by the Phenol Red-, steroid- and/or keoxifene-treated cultures was expressed in terms of percentage of the LH release by the respective vehicle-treated cultures (no Phenol Red, estradiol or keoxifene = 100%). The data obtained in three or four independent experiments run in triplicate each were pooled. Statistical analysis was performed as follows: first the data were tested for homogeneity of variance using the Bartlett-test. Then analysis of variance (ANOVA) was carried out. Statistical differences between individual groups were determined by the Newman–Keuls-test. If variances were not homogeneous a Kruskal–Wallis-test was

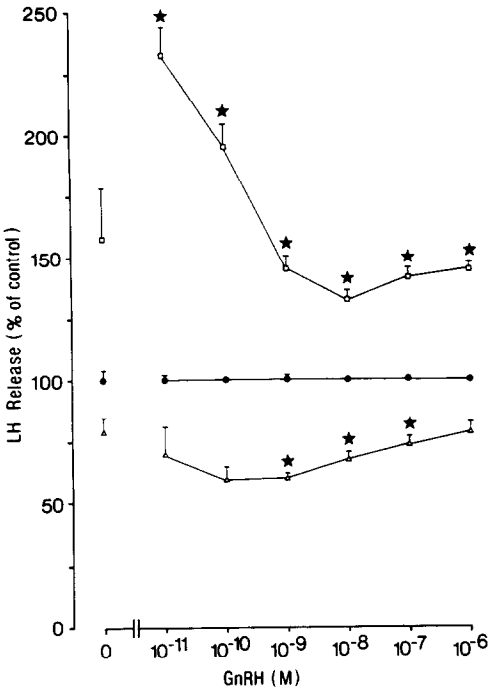

Fig. 1. Effects of short-term and long-term treatment of pituitary cell cultures with estradiol (E_2). Cell cultures were incubated in Phenol Red-free M 199 and treated for 4 (△) or 24 h (□) with 1 nM E_2. During the last 3 h of incubation the cells were stimulated with increasing concentrations of GnRH. The data from four independent experiments run in triplicate each were pooled and are expressed (mean ± SE) as percentage of LH release by the respective control cultures [●, vehicle (V)]. Mean absolute LH values corresponding to 100% are 2.3 ± 0.4 ng/ml at 0; 8.8 ± 1.8 ng/ml at 10 pM; 15.5 ± 2.6 ng/ml at 100 pM; 25.8 ± 1.9 ng/ml at 1 nM; 37.6 ± 2.6 ng/ml at 10 nM; 41.9 ± 3.5 ng/ml at 100 nM and 38.9 ± 2.9 ng/ml at 1 μM GnRH. $^*P < 0.05$ vs V (Newman–Keuls-test); the P value of ANOVA is <0.05.

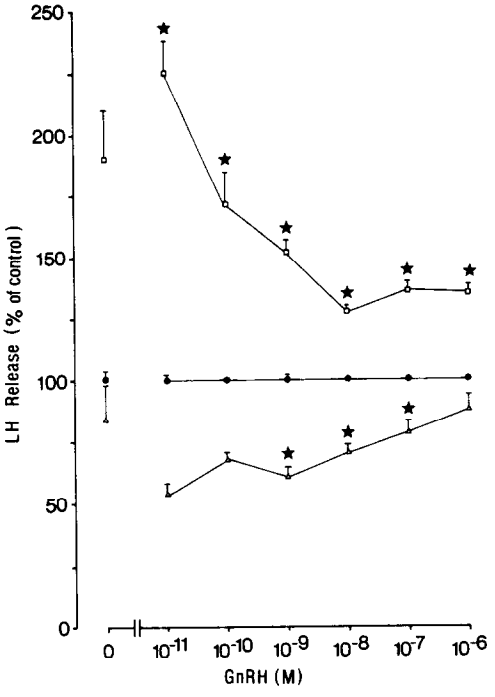

Fig. 2. Effects of short-term and long-term incubation of pituitary cell cultures with M 199 containing Phenol Red. Cell cultures were incubated for 4 (△) or 24 h (□) with regular M 199 containing 50 μM Phenol Red and stimulated with increasing concentrations of GnRH during the last 3 h of incubation. Respective control cultures [●, vehicle (V)] were incubated in Phenol Red-free medium 199. For further details and mean absolute LH values in control cultures see Fig. 1. $^*P < 0.05$ vs V (Newman–Keuls-test); the P value of ANOVA is <0.05.

performed followed by a Nemenyi-test for comparison of individual groups.

RESULTS

Short-term and long-term actions of Phenol Red and estradiol on LH secretion

Short-term incubation (4 h) of pituitary cells with medium 199 containing Phenol Red or 1 nM estradiol led to reduced LH responses to increasing concentrations of GnRH. Maximal effects were observed at 1 nM GnRH a dose which is close to the ED_{50} of the decapeptide. At this concentration of GnRH Phenol Red (50 μM) and estradiol (1 nM) suppressed LH secretion by 39 and 38% respectively (Figs 1 and 2). Long-term incubation (24 h) of cell cultures with either Phenol Red or estradiol resulted in the contrary effect i.e. enhancement of LH secretion. After this treatment maximal relative enhancement was observed at the lower concentrations (10 pM, 100 pM) of GnRH, also basal LH secretion was significantly increased (Figs 1 and 2).

Effects of increasing concentrations of Phenol Red on LH secretion

When pituitary cells were treated for 4 h with increasing concentrations (100 pM–1 mM) of Phenol Red and stimulated with a submaximal dose of GnRH (1 nM), their LH response was inhibited at concentrations ⩾ 10 μM. 24 h treatment with Phenol Red resulted in enhanced LH responses to the GnRH stimulus at concentrations ⩾ 10 μM with a maximal effect at 100 μM, an effect which was lost at 1 mM Phenol Red resulting in a bell-shaped dose–response curve (Fig. 3).

Effects of keoxifene on long-term and short-term actions of Phenol Red and estradiol on LH secretion

4 h treatment of pituitary cells with 100 μM Phenol Red or 1 nM estradiol led to reduced LH responses after GnRH stimulation. In the presence of 100 nM keoxifene the inhibitory effects of both estradiol and Phenol Red were blocked resulting in LH responses that were indistinguishable from those observed in control cultures. When the cell cultures were treated for 24 h with Phenol Red or estradiol and stimulated with 1 nM GnRH LH secretion was significantly enhanced by 55 and 46% respectively. Addition of

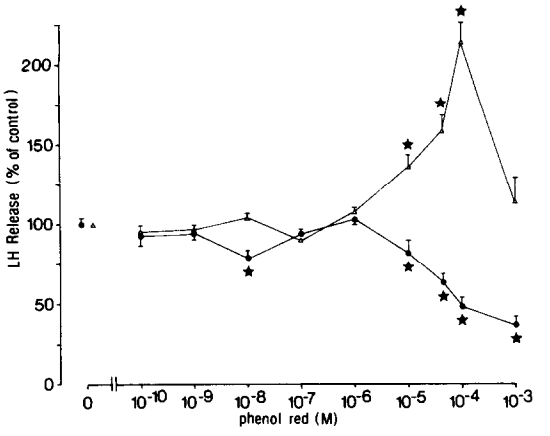

Fig. 3. Effects of increasing concentrations of Phenol Red on GnRH-induced LH release. Cell cultures were treated for 4 (●) or 24 h (△) with 100 pM–1 mM Phenol Red and stimulated for 3 h with 1 nM GnRH. Mean absolute LH values of three independent experiments corresponding to 100% are 19.1 ± 1.4 ng/ml at 4 h and 12.4 ± 1.5 ng/ml at 24 h. For further details see Fig. 1. $*P < 0.05$ vs V (Newman–Keuls-test); the P value of ANOVA is <0.05.

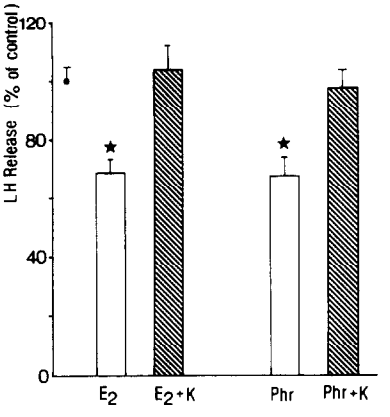

Fig. 5. Effects of keoxifene (K) on the short-term inhibitory action of estradiol (E_2) and Phenol Red (Phr) on GnRH-induced LH secretion. Cell cultures were incubated for 4 h with 1 nM E_2 or 100 μM Phr without □ or with ▨ 100 nM keoxifene, and stimulated for 3 h with 1 nM GnRH. The mean absolute LH value of three independent experiments corresponding to 100% is 13.6 ± 5.2 ng/ml. (●) indicates the control. For further details see Fig. 1. $*P < 0.05$ vs V (Newman–Keuls-test); the P value of ANOVA is <0.05.

100 nM keoxifene to such cell cultures resulted in LH responses to GnRH that were not different from those of vehicle-treated cultures (Figs 4 and 5).

Effects of increasing concentrations of keoxifene on LH secretion of pituitary cells cultivated in medium 199 with or without Phenol Red

When cells that were cultivated in medium 199 containing Phenol Red were treated with increasing concentrations of keoxifene (1 pM–10 μM) the GnRH-stimulated LH secretion was significantly reduced at concentrations ⩾10 nM. The maximal suppression was achieved at 10 μM where gonadotrophin secretion was reduced by 51%. If the same experiment was performed in Phenol Red-free medium 199 the negative effect of keoxifene could only be observed at the highest concentration (10 μM) where LH secretion was reduced by 28% (Fig. 6).

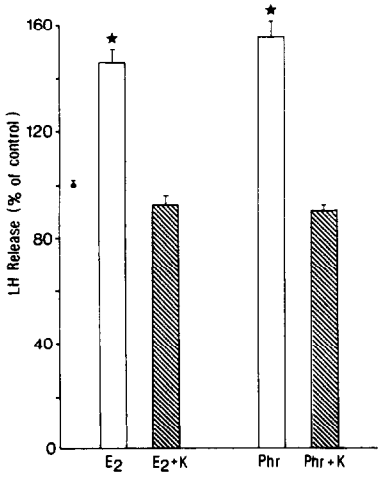

Fig. 4. Effects of keoxifene (K) on the long-term stimulatory action of estradiol (E_2) and Phenol Red (Phr) on GnRH-induced LH secretion. Cell cultures were incubated for 24 h with 1 nM E_2 or 100 μM Phr without □ or with ▨ 100 nM keoxifene, and stimulated for 3 h with 1 nM GnRH. The mean absolute LH value of three independent experiments corresponding to 100% is 22.7 ± 3.4 ng/ml. (●) indicates the control. For further details see Fig. 1. $*P < 0.05$ vs V (Nemenyi-test); the P value of the Kruskal–Wallis-test is <0.001.

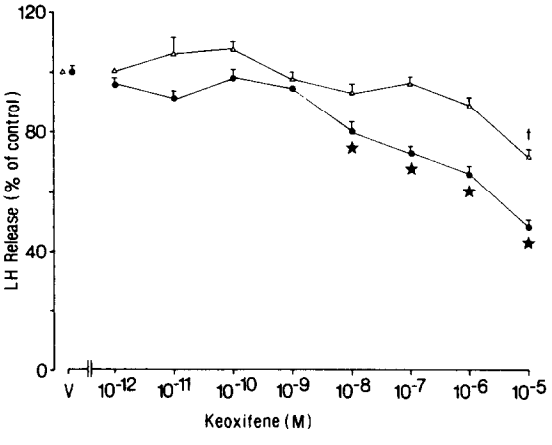

Fig. 6. Effects of increasing concentrations of keoxifene on GnRH-induced LH release. Cell cultures were incubated for 24 h with 1 pM–10 μM keoxifene in the absence (△) or presence of Phenol Red (50 μM) (●) and stimulated for 3 h with 1 nM GnRH. Mean absolute LH values of three independent experiments corresponding to 100% are 27.0 ± 7.4 ng/ml when the cells were cultured in Phenol Red-free M 199 and 37.9 ± 4.7 ng/ml when the cells were cultured in M 199 containing Phenol Red. $*P < 0.05$ vs V (Newman–Keuls-test); the P value of ANOVA is $P < 0.05$. †$P < 0.05$ vs V (Nemenyi-test); the P value of the Kruskal–Wallis-test is <0.001.

DISCUSSION

The present study clearly demonstrated that two different preparations of the pH indicator Phenol Red possess estrogenic activity in the rat gonadotroph. There were no differences in potency between the two preparations tested here. It was shown that long-term treatment of pituitary cells with Phenol Red increased their sensitivity to GnRH, while short-term treatment led to reduced LH responses after GnRH stimulation. These time-dependent inhibitory and stimulatory effects on gonadotrophin secretion are well established actions of estradiol [10, 11]. As we intended to check if these effects are also present when Phenol Red is omitted from the culture medium we carried out experiments in which pituitary cells were cultivated for 4 or 24 h with 1 nM estradiol in medium 199 without Phenol Red and stimulated with GnRH during the last 3 h of the incubation periods. In parallel another set of cell cultures was treated with Phenol Red instead of estradiol. The results of these experiments show a striking similarity (Figs 1 and 2). Both estradiol and Phenol Red led to inhibition after short-term or enhancement of LH release after long-term treatment and the results correspond not only qualitatively but also quantitatively. If we compare our present data on positive and negative estrogen actions to those of previous experiments we have to consider that there are no differences concerning the stimulatory action of estrogen but the inhibitory action is often more pronounced and seems to be more consistent in the experiments in which we used Phenol Red-free medium. This possibly explains why some authors could not demonstrate an inhibitory action of estradiol on LH secretion [9].

We also investigated the dose–response characteristics of Phenol Red. It was shown that concentrations $\geqslant 10\,\mu$M of Phenol Red were able to either reduce (4 h incubation) or enhance GnRH-stimulated LH release. Phenol Red concentrations in regular medium 199 vary between 40 and 50 μM and were shown to be fully effective. Interestingly the positive effect of Phenol Red which was observed after long-term treatment and became maximal at 100 μM was lost when we used the concentration of 1 mM, resulting in a bell-shaped dose–response curve. Such bell-shaped dose–response curves are characteristic for estrogens and have recently been described by our group [18]. Obviously the concentrations of Phenol Red required to induce a stimulatory effect on LH secretion are much higher than those of estradiol. The difference in potency between the two compounds is about four orders of magnitude.

Apart from the qualification of the Phenol Red effects we wanted to determine their specificity by the use of the antiestrogen keoxifene. By this means we were able to antagonize both the positive and negative action of Phenol Red on LH secretion supporting the assumption that these actions are mediated via the estrogen receptor. Although our data clearly demonstrate estrogenic effects of two different Phenol Red preparations this finding could not always be confirmed by others. Recently Bindal *et al.* purified Phenol Red preparations from different origins and showed that not phenolsulfonphthalein but lipophilic impurities within these preparations induced estrogenic effects [8]. The same authors were able to isolate the estrogenic component from a Phenol Red preparation and identified it spectroscopically as bis(4-hydroxyphenyl)[2-(phenoxy-sulfonyl)phenyl]methane [19]. Differences in contamination of Phenol Red preparations might explain why most but not all authors were able to show estrogenic effects of Phenol Red in different tissues.

In a recent paper we have described that keoxifene exerts an inhibitory effect on GnRH-stimulated LH secretion and have discussed its possible mechanism [15]. Such inhibitory actions are well known for other antiestrogens of the triphenylethylene type. Berthois *et al.* have shown that tamoxifen which inhibits growth of MCF-7 cells in the absence of estrogen are free of such activity when the cells were cultivated in Phenol Red-free medium [1]. Thus it might be possible that the inhibitory effect of keoxifene on LH secretion is due to antagonism of the estrogenic activity of the Phenol Red preparation that was present in the culture medium. Therefore we repeated our experiment under conditions in which Phenol Red was omitted. When we used medium containing Phenol Red the inhibitory effect was present at concentrations $\geqslant 10$ nM while it could only be observed at 10 μM when the dye was excluded. Thus the inhibitory effect of keoxifene seems to be partly but not exclusively due to antagonism of the estrogenic activity of the Phenol Red preparation.

We conclude that the established effects of estradiol on LH secretion are valid as we were able to reproduce them in Phenol Red-free medium. The two Phenol Red preparations tested in this study exerted actions which were qualitatively undistinguishable from estrogen actions and seem to be mediated by interaction with the estrogen receptor. Keoxifene's inhibitory action in the gonadotroph canot be fully explained by antagonism of the estrogenic activity of Phenol Red. We recommend that Phenol Red should be omitted from the culture media when estrogen sensitive cells or tissues are used especially when estrogen or antiestrogen actions are investigated.

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