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Original article

Effects of 17β-estradiol on calcitonin and calcitonin-gene-related peptide secretions and contents in a murine medullary thyroid carcinoma C-cell line (CA-77)

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Summary — The effect of 17β-estradiol on calcitonin (CT) and calcitonin-gene-related peptide (CGRP) secretions in the murine CA-77 C cell line was studied after 1, 3, 5 and 6 d of treatment. The release of both CT and CGRP significantly increased 1, 3, 5 and 6 d after addition of 0.1 μmol/l estradiol alone to the culture medium. The C cell content of both peptides also increased after 6 d of treatment with the same dose of estrogen. The enhanced CT and CGRP secretions induced by 17β-estradiol were not inhibited by the simultaneous addition of 5 μmol/l of all-*trans*-retinoic acid. Dexamethasone alone increased the release of both peptides within 6 d. However, when cells were treated simultaneously with estradiol and 1 μmol/l dexamethasone, the addition of retinoic acid blunted both the CT and CGRP secretions induced by dexamethasone. These results showed that the positive effects of 17β-estradiol on both CT and CGRP secretions were modulated by dexamethasone and retinoic acid.

CT secretion / CGRP secretion / CA-77 C cell / 17β-estradiol / dexamethasone / retinoic acid

Résumé — Le 17β-œstradiol à la dose de 100 nmol/l augmente la sécrétion et le contenu cellulaire de CT et de CGRP par la lignée de cellules C (CA-77) d'origine murine. *Les effets de l'œstradiol sur la libération de CT et de CGRP dans le milieu de culture sont observés 1, 3, 5 et 6 j après addition du stéroïde. Le contenu cellulaire en hormones est également légèrement augmenté sous œstradiol. L'acide rétinoïque all-*trans *5 μmol/l ne diminue la sécrétion des 2 peptides qu'au bout de 6 j, alors que la dexaméthasone 1 μmol/l, seule, stimule la sécrétion de CT et CGRP tout au long du traitement (de 1 à 6 j). Si l'acide rétinoïque n'affecte pas le contenu cellulaire en CT et CGRP, la dexaméthasone, en revanche, l'augmente. Le co-traitement par l'œstradiol et l'acide rétinoïque n'inhibe pas l'effet du stéroïde sur la sécrétion de CT et de CGRP. Dans le cas d'un co-traitement par la dexaméthasone et l'œstradiol, un effet additif n'est observé qu'après 3 j de traitement. Le traitement simultané des cellules C par les 3 molécules montre à chaque fois (1, 3, 5 et 6 j) que l'acide rétinoïque inhibe l'induction de la*

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libération des 2 peptides observée lors du traitement combiné par l'œstradiol et la dexaméthasone. Nos résultats suggèrent que les œstrogènes augmentent la sécrétion de CT et de CGRP et que cet effet peut être modulé par les glucocorticoïdes et l'acide rétinoïque.

CT / CGRP / cellules C / œstrogènes / glucocorticoïdes / acide rétinoïque

INTRODUCTION

Estrogen therapy has been used to inhibit bone resorption and therefore to prevent or to treat osteoporosis in postmenopausal women (Genant *et al,* 1983). It remains controversial whether this estrogen effect occurs directly at the bone level only or whether it is at least partially mediated *via* the stimulation of calcitonin (CT) secretion. It was recently suggested that osteocytes are major skeletal estrogen target cells in 3 different species (Braidman *et al,* 1995). One possible mediating hormone is CT, which, by reducing bone resorption, could prevent or reduce osteoporosis. Several investigators have shown that the circulating concentrations of CT are decreased in the oophorectomized or postmenopausal state (Milhaud *et al,* 1978), and that estrogen increases CT secretion (Morimoto *et al,* 1980). In oophorectomized rats, the plasma CT response to a calcium challenge is impaired and can be restored to normal levels by estrogen replacement therapy (Catherwood *et al,* 1983). Rat pup thyroid incubation studies demonstrate that estradiol can stimulate CT secretion *in vitro* (Greenberg *et al,* 1986). In contrast, in the human C cell carcinoma cell line (TT) with well-documented estrogen receptors, estradiol induces a dose-dependent inhibition of CT secretion (Lazaretti-Castro *et al,* 1991). Since estrogen receptors were recently detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in rat C cells (Naveh-Many *et al,* 1992), we investigated the effects of 17β-estradiol on CT and calcitonin-gene-related peptide (CGRP) secretion in the murine C cell line (CA-77). The interaction of glucocorticoids and retinoic acid on the effects of estrogen was also analysed.

MATERIALS AND METHODS

Cell culture methodology

The Ca-77 cells were maintained in Dulbecco modified Eagle's medium (DMEM)/Ham's F10 (1:1) (Gibco/BRL, France) supplemented with 10 μg/ml insulin (Sigma), 3 x 10^{-8} M sodium selenite (Gibco/BRL, France), 5 μg/ml transferrin (Gibco/BRL, France), 110 mg/l sodium pyruvate (Gibco/BRL, France), and a mixture of antibiotics (100 units/ml penicillin + 100 μg/ml streptomycin; Gibco/BRL, France). Cells were plated at an initial density of 4 x 10^4 cells/cm^2 in a medium containing DMEM/Ham's F10 (1:1), 10% heat-inactivated fetal calf serum (J Boy, Reims, France), 110 mg/l sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 48 h of plating, the medium was changed and replaced by the growth medium described above; the growth medium with fresh hormone was changed each day. Cells were continuously exposed to the hormones. Dexamethasone (Sigma), all-*trans*-retinoic acid (Sigma), and 17β-estradiol (Sigma) were dissolved in absolute ethanol and then in phosphate-buffered saline (PBS) to reach a final ethanol concentration of 0,005% in the growth medium. Controls with the same volume of vehicle were used in each case.

Other methods

Cell culture medium was collected and treated as previously described (Wind *et al,* 1993) to measure the CT and CGRP levels. The peptide contents (CT and CGRP) of C cells were also measured by radioimmunoassay (RIA); 400 μl

0.1 N HCl was added to culture dishes. The cells were then scraped and sonicated for 15 s and allowed to stand for 16 h at 4°C. The CT RIA has been reported previously (Heath and Sizemore, 1982; Jousset *et al,* 1988). The G813 antibody (goat antiserum raised against synthetic human CT) was a gift of Dr H Heath (Mayo Clinic and Mayo Foundation, Rochester, MN, USA), and the detection limit of the assay was 3.9 pg per tube. This assay used ^{125}I-labeled human CT and unlabeled synthetic human CT (Ciba-Geigy, Basel, Switzerland) as standards. The results are expressed as nanogram equivalent human CT/mg protein since the protein content of culture dishes was determined by a Lowry method (Lowry *et al,* 1951). Intra-assay variations for the CT RIA were 5% and inter-assay variations were 10%. For the CGRP RIA, we used synthetic human CGRP (Sigma) as a standard, a sheep antibody raised against synthetic human CGRP (kindly donated by Dr MS Moukthar, Unité INSERM 349, Paris) diluted 1:100 000, and ^{125}I-iodohistidyl human CGRP from Amersham (Les Ulis, France). The tubes were preincubated for 4 d at 4°C and then incubated for 3 d at 4°C in presence of labeled CGRP. The bound and free fractions were separated by adding 0.15 ml of dextran charcoal suspension buffer to each assay tube (0.5 ml); after centrifugation at 2 000 *g* for 20 min, the supernatant was discarded. The detection limit of this assay was 10 pg per tube.

All probabilities were calculated using the Mann-Whitney *U*-test for ranked non-parametric data.

RESULTS

After 1 d of treatment with 10^{-7} mol/l 17β-estradiol, the release of CT and CGRP increased (figs 1 and 2), the C cell content in CT was also elevated above the control values (table I). The secretion of both CT and CGRP under estrogen treatment remained higher than controls within 6 d (figs 1 and 2) (1 494 ± 103 *versus* 1 093 ± 73 pg CT/mg protein in controls, $P < 0.05$, and 1 220 ± 50 *versus* 830 ± 38 ng CGRP/mg protein in controls, $P < 0.001$ after 5 d of estrogen). If the cellular content of peptides (CT and CGRP) was still increasing on day 6 of estrogen treatment, the release in the culture medium of the 2 peptides was slightly above the controls (figs 1 and 2). As expected from previous results (Lamari *et al,* 1994), an increase in both the cellular content and the release

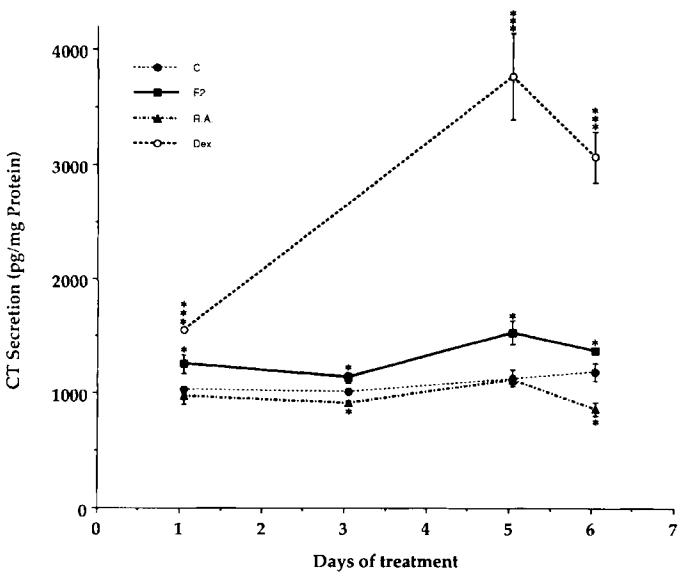

Fig 1. Effect of 17β-estradiol (E2), retinoic acid (RA) and dexamethasone (Dex) on CT release by CA-77 C cells. Medium containing fresh hormone was changed each day and cells were continuously exposed in the presence of hormones. C: controls, E2: 0.1 μmol, RA: 5 μmol/l all-*trans*-retinoic acid, Dex: 1 μmol/l. Means ± SEM of 4 culture dishes. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ from controls.

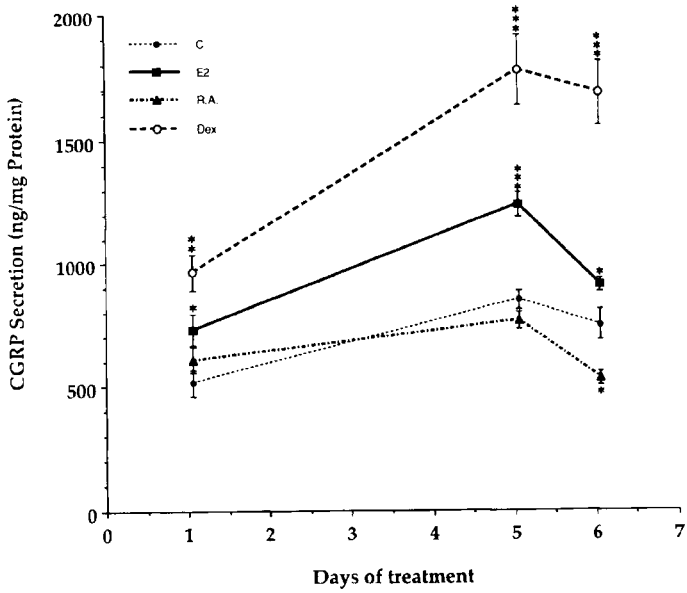

Fig 2. Effect of 17β-estradiol (E2), retinoic acid (RA) and dexamethasone (Dex) on CGRP release by CA-77 C cells. Medium containing fresh hormone was changed each day and cells were continuously exposed in presence of hormones. C: controls, E2 0.1 μmol/l, RA: 5 μmol/l all-*trans*-retinoic acid, Dex: 1 μmol/l. Means ± SEM of 4 culture dishes. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ from controls.

in the medium of CT and CGRP occurred after 10^{-6} mol/l dexamethasone treatment (figs 1 and 2). The release of both CT and CGRP was only higher after 3 d of simultaneous treatment with the same doses of estradiol and dexamethasone than when the C cells were exposed to dexamethasone alone (1 750 ± 164 *versus* 1 285 ± 29 pg CT/well in the Dex group, $P < 0.05$; and 1 447 ± 60 *versus* 1 200 ± 52 ng CGRP/well in the Dex group, $P < 0.05$). An additive effect for the C cell content in CT was observed 1 d after treatment with both dexamethasone and estradiol (table I) in

Table I. Cellular content in CT and CGRP after treatment of CA-77 C cells.

	1 d CT (pg/mg protein)	*1 d CGRP (ng/mg protein)*	*6 d CT (pg/mg protein)*	*6 d CGRP (ng/mg protein)*
Controls	542 ± 12	329 ± 18	525 ± 32	310 ± 14
E2	693 ± 47[a]	283 ± 22	638 ± 25[a]	346 ± 10[a]
Retinoic acid	524 ± 39	354 ± 18	588 ± 33	386 ± 10[b]
Dex	735 ± 22[c]	395 ± 13[a]	1 348 ± 80[c]	579 ± 28[c]
E2 ± retinoic acid	968 ± 26[d]	378 ± 11[d]	1 006 ± 65[d]	638 ± 31[e]
E2 + Dex	1 082 ± 29[e]	424 ± 21[d]	1 336 ± 91[e]	538 ± 19[e]
E2 + Dex + retinoic acid	1 014 ± 65	409 ± 12	1 095 ± 89	461 ± 38

Means ± SEM of 4 culture dishes. [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ from controls. [d] $P < 0.01$, [e] $P < 0.001$ from E2 group. Medium containing fresh hormone was changed each day and cells were continuously exposed in the presence of hormones. E2 0.1 μmol/l, retinoic acid: 5 μmol/l all-*trans*-retinoic acid, Dex: 1 μmol/l.

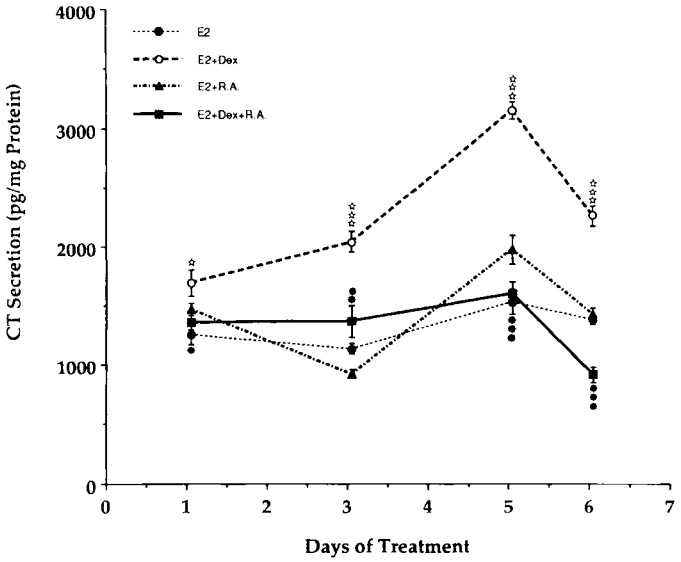

Fig 3. Effect of 17β-estradiol (E2), E2 + retinoic acid (RA), E2 + dexamethasone (Dex), and E2 + Dex + RA on calcitonin (CT) release by Ca-77 C cells. Medium containing fresh hormone was changed each day and cells were continuously exposed in presence of hormones. E2: 0.1 μmol/l, RA: 5 μmol/l all-*trans*-retinoic acid, Dex: 1 μmol/l. Means ± SEM of 4 culture dishes. ☆☆ $P < 0.01$, ☆☆☆ $P < 0.001$ from E2 group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ from E2 + Dex group.

comparison to the dexamethasone group. On day 6 of treatment, 5 x 10^{-6} mol/l all-*trans*-retinoic acid clearly decreased the release of both CT and CGRP (figs 1 and 2). As compared to the effects in cells co-treated with estradiol and dexamethasone, a decrease in the secretion of both CT and CGRP occurred when the cells were exposed to the simultaneous action of estradiol, dexamethasone, and all-*trans*-retinoic acid (figs 3 and 4). This was clearly observed from days 1 to 6 of treatment,

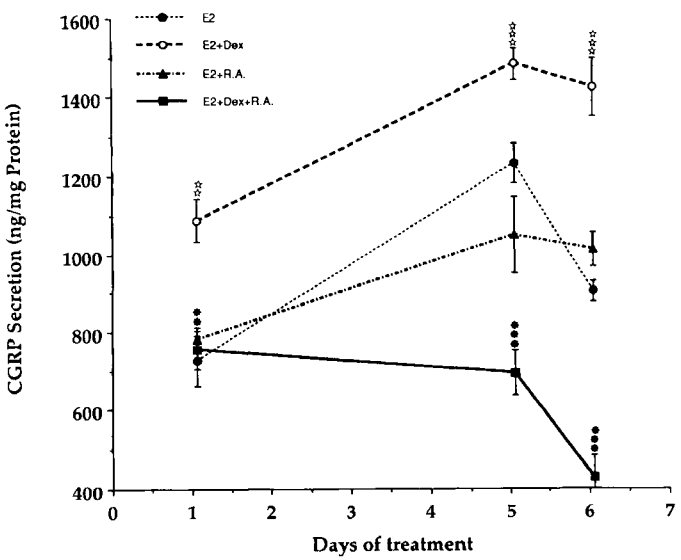

Fig 4. Effect of 17β-estradiol (E2), E2 + retinoic acid (RA), E2 + dexamethasone (Dex), and E2 + Dex + RA on CGRP release by CA-77 C cells. Medium containing fresh hormone was changed each day and cells were continuously exposed in presence of hormones. E2: 0.1 μmol/l, RA: 5 μmol/l all-*trans*-retinoic acid, Dex: 1 μmol/l. Means ± SEM of 4 culture dishes. ☆☆ $P < 0.01$, ☆☆☆ $P < 0.001$ from E2 group. ** $P < 0.01$, *** $P < 0.001$ from E2 + Dex group.

but the addition of all-trans-retinoic acid did not change the elevated C cell contents of both peptides induced by estradiol plus dexamethasone (table I).

DISCUSSION

The C cell carcinoma cell lines offer good model systems for studying the direct effects of estrogens on CT secretion because they express estrogen receptors (Yang *et al,* 1988). The dose of 17β-estradiol used in the present *in vitro* studies (100 nmol/l) was shown to achieve a maximal increase of CT secretion by thyroparathyroid gland complexes of 8-d-old rat pups (Greenberg *et al,* 1986), even plasma 17β-estradiol concentrations in female rats is approximately 1 nmol/l. Although for cell culture, 100 nmol/l of estrogens is routinely used by most authors, this concentration appeared more pharmacological than physiological.

The use of the human TT C cell line has shown no stimulatory effect due to estrogen on spontaneous CT secretion and CT content, but a transient inhibitory effect with a nadir after 24 h (Lazaretti-Castro *et al,* 1991). In contrast, in our present results, 17β-estradiol stimulated both CT and CGRP secretion in the murine CA-77 C cell line after a long-term exposure (from day 1 up to day 6). These data are in agreement with the *in vivo* observations in female rats that show the presence of estrogen receptors in C cells by PCR (Naveh-Many *et al,* 1992), and also with the fact that estradiol given to ovariectomized animals prevents the decrease in CT mRNA levels (Naveh-Many *et al,* 1992; Lamari *et al,* 1994). Thus, C cells are target organs for estrogens, and stimulated CT secretion appears to result from increased steady-state CT mRNA levels leading to an increased biosynthesis of the hormone. As shown by our results, the C cell content of CT and CGRP increased under the estrogen treatment of murine CA-77 C cells. Ovariectomy produces osteopenia in female rats, which can be impaired by estrogen therapy (Takano-Yamamoto and Rodan, 1990; Kalu *et al,* 1991). The present study suggests that estrogens act on bone to prevent osteoporosis not only by a direct action on osteoblasts and osteocytes but also indirectly by its action on C cells.

The fact that the synthetic glucocorticoid dexamethasone stimulates CT gene expression (Russo *et al,* 1988; Collignon *et al,* 1992) and enhances the biosynthesis and release of both CT and CGRP (Lamari *et al,* 1994) in CA-77 C cells has already been reported. All-*trans*-retinoic acid also was shown to decrease both CT and CGRP mRNAs in CA-77 C cells and the secretion of both peptides (Lamari *et al,* 1994). The present results suggest a possible interplay between dexamethasone, estrogen and retinoic acid on CT and CGRP secretion by the CA-77 C cell line.

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