Evidence that corticotropin-releasing hormone inhibits cell growth of human breast cancer cells via the activation of CRH-R1 receptor subtype

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Abstract

It has been previously shown that corticotropin-releasing hormone (CRH) exerts antiproliferative activity on an estrogen-dependent tumor cell line, i.e. human endometrial adenocarcinoma Ishikawa (IK) cells. Here we have investigated the effects of CRH on another estrogen-dependent tumor cell line, human breast cancer MCF7 cells. In this paradigm, CRH given at a fixed concentration of 100 nM significantly inhibited cell growth induced by 100 nM estradiol (E2) after 48 and 72 h of incubation. This effect was not associated with the induction of apoptosis. CRH inhibition of cell proliferation was counteracted in a concentration-dependent manner by the non-selective CRH receptor antagonist, astressin, as well as by a CRH-R1 selective receptor antagonist, antalarmin. RNase protection assays carried out on MCF7 under basal conditions showed that these cells express in a constitutive manner the CRH-R1 receptor subtype. We have also investigated the putative source of CRH acting on breast cancer cells; we found that MCF7 cells express CRH mRNA under basal conditions and secrete sizable amounts of immunoreactive CRH, which leads to postulate the existence of paracrine–autocrine inhibitory mechanism operated by CRH in breast cancer cells.

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1. Introduction

The role of corticotropin-releasing hormone (CRH) and its receptors in the modulation of tumor proliferation has been thoroughly investigated in the last years (Bale et al., 2002; Reubi et al., 2003). In light of the crucial role exerted by the peptide in the regulation of normal endometrial function (Florio et al., 2003), we have previously investigated the effects of CRH on cell proliferation in a human endometrial tumor cell line, Ishikawa (IK) cells. We found that CRH inhibits both basal and estrogen-stimulated IK cell proliferation via the binding and activation of CRH-R1 receptors (Graziani et al., 2002, 2006).

Having observed such interesting effect of CRH on an estrogen-dependent tumor line, we have also considered that another estrogen-dependent tumor, namely breast cancer, represents a more relevant clinical condition, in terms of both higher incidence and poorer prognosis, compared to endometrial cancer (Edwards et al., 2005). Therefore, in the present study we searched for possible antiproliferative effects of CRH in a human breast cancer cell line expressing estrogen receptors, MCF7 cells. CRH gene expression and protein biosynthesis, as well as the gene expression of CRH type-1 receptor were also investigated in these cells.

2. Materials and methods

2.1. Cell line and culture conditions

The human breast cancer cell line MCF-7 (ATCC-LGC Promochem, Milan, Italy) and the human endometrial adenocarcinoma Ishikawa cell line (kindly provided by Profs. Gigliola Sica, Institute of Histology, Catholic University Medical School, Rome) were cultured in DMEM (Sigma–Aldrich, Milan, Italy) supplemented with 10% fetal calf serum (Sigma), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Flow Laboratories, Mc Lean, VA), at 37 °C in a 5% CO2 humidified atmosphere. The human B lymphoblastoid MT-1 cell line (a generous gift of W.G. Thilly, Massachusetts Institute of Technology,
Cambridge, MA) were cultured in RPMI-1640 (Sigma–Aldrich) supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics.

In experiments looking at interaction with estrogens, cells were cultured in phenol red-free DMEM (Gibco), in order to remove estrogenic components present in the serum and estrogenic contaminants in phenol red indicator, supplemented with charcoal/dextran-stripped FBS (Hyclone, Logan, UT, USA), 1 mM sodium pyruvate and antibiotics.

2.2. Drug treatment

Estradiol (E2, Sigma–Aldrich) was dissolved in 95% ethanol at the concentration of 10^{-3} M. CRH stock solution (10^{-5} M) was prepared in 0.01N HCl/0.1% bovine serum albumin (BSA) in PBS (vehicle). Astressin was dissol-ved in 0.01 M HCl/0.1% BSA in distilled water; antalarmin stock solution (5 × 10^{-4} M) was prepared in 6.6% dimethylsulfoxide and 3.3% chremophor solution. Both drugs were diluted to working concentration in incubation medium.

Five days before starting an experiment, cells were cultured in phenol red-free DMEM supplemented with 10% heat-inactivated charcoal/dextran-stripped FBS. Thereafter, cells were plated in flasks (1.5 × 10^6 cells/flask) in phenol red-free DMEM culture medium containing 1% charcoal/dextran-stripped FBS. Cells were then treated with vehicle or with E2, given at concentrations ranging from 1 to 100 nM either in the absence or in the presence of 1–100 nM CRH (Sigma–Aldrich). Cell counts were carried out as previously described after 48 and 72 h of incubation and cell viability was determined by trypan blue dye exclusion.

2.3. Microscopic analysis of CRH effects on MCF-7 cells vitality

The effects of CRH on MCF-7 cells vitality were analyzed through the use of fluorescence microscopy with the nuclear dye Hoechst 33258, as previously described (Pozzoli et al., 2006). Briefly, cells were plated at a density of 60,000 cells per 35 mm dish containing glass coverslips. After exposure to vehicle (control) or 100 nM CRH for 48 h, cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were then washed three times with PBS and incubated for 10 min in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37 °C with the DNA-binding dye Hoechst 33258 (0.5 μg/ml in PBS). After being washed with water, coverslips were mounted onto glass slides and photographed on a Nikon (Tokio, Japan) Eclipse TE300 fluorescence inverted microscope with excitation at 360 nm, at a magnification of 400×.

Cells were considered viable if their chromatin was diffuse and evenly distributed throughout the nucleus. Each condition was represented in two dishes per experiment.

2.4. RNase protection analysis

Total RNA was extracted by the guanidine thiocyanate lysis method of Chomczynski and Sacchi (1987). To measure CRH-R1 mRNA expression, a plasmid (pGP-1) containing a 522-bp fragment of the human CRH-R1 cDNA encoding the N-terminal 174 amino acids (Graziani et al., 2002) was linearized with Sty1, and an antisense riboprobe of 244-nucleotides, as an internal loading control. The hGAPDH plasmid (pTRI, Ambion, Austin, CA) were cultured in RPMI-1640 (Sigma–Aldrich) supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics.

Total RNA was treated with RNase-free DNase and cDNA was synthesized by incubating 1.5 μg RNA with 0.5 U of AMV RT and 0.2 μg oligo(dT) primer at 42 °C for 1 h, using the cDNA Cycle® Kit from Invitrogen. Five microlitre aliquots of the reverse-transcribed cDNA were subjected to 35 cycles of PCR in 50 μl of 1× buffer (10 mM Tri–HCl, pH 8.3, 1.5 mM MgCl_2, 50 mM KCl) containing 1 mM each of dATP, dCTP, dGTP and dTTP, 2.5 U of Taq DNA polymerase (Roche, Milan, Italy) and 0.2 nM of CRH specific primers (5′-TCCGAGGAGCTCCATCCATC-3′ and 5′-AATCTCCATGAGTTTCCGTGTC-3′; GeneBank accession number: NM_031019), which amplify a fragment of 122 bp. For amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1 μl aliquot of the reverse-transcribed cDNA was amplified using the following primers: 5′-TGGTATCGTGAAGGACTCATGAC-3′ and 5′-ATGCCAGTGACGTTCGGCTACG-3′ (GeneBank accession number: BC023632), which amplify a 190 bp product. Each cycle consisted of denaturation at 94 °C for 45 s, annealing at 60 °C for 90 s and extension at 72 °C for 90 s.

Twenty microlitres of PCR reaction were electrophoresed through a 2% agarose gel containing ethidium bromide.

2.5. RT-PCR

Fig. 1. Corticotrophin-releasing hormone significantly inhibits MCF7 cell proliferation induced by 100 nM E2 after 48 (panel A) and 72 h (panel B) of incubation. Experiments carried out in the absence (empty circles) and in the presence (full circles) of 100 nM CRH. Each symbol value represents the mean of cell counts performed in quadruplicate. Bars: S.E.M. The results are representative of one out of three repeated experiments. Symbol ‘*‘: p < 0.01 vs. E2 alone.
above. At the end of experiments, incubation media were collected and stored at −80 °C until assayed for CRH immunoreactivity. Cells were re-suspended in 250 μl of Tris–HCl buffer (50 mM solution at pH 7.4 containing 0.2% bovine serum albumin and 40 IU/ml aprotinin) and sonicated for 30 s with a Labsonic sonicator (B. Braun Biotech International, Melsungen, Germany). After 15-min centrifugation at 14,000 rpm and 4 °C, supernatants were collected and assayed for CRH immunoreactivity.

CRH was measured by radioimmunoassay (RIA) as previously described (Navarra et al., 1991), with the following modifications: an CRH antiserum (kindly donated by Prof. R. Bernardini) and (2-[125I]-iodohystidyl32)CRF (Amersham-Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) were used. The detection limit of the assay was 1 pg/tube (100 μl sample volume for incubation media), with intra- and inter-assay coefficients of variation of 5 and 10%, respectively.

2.7. Statistical analysis

Data were expressed as individual values or the means ± 1 standard error of the mean (S.E.M.) of (n) replicates per group. Data were analyzed by ANOVA and post hoc Newman–Keul test for multiple comparisons among group means, or by Student’s t-test where appropriate, using a Prism™ computer program (GraphPad, San Diego, CA). Differences were considered statistically significant if p < 0.05.

3. Results

As expected, E2 given in the range 1–100 nM caused a concentration-dependent increase in MCF7 cell proliferation after 48- and 72-h incubations (Fig. 1A and B). Similar to experiments on IK cells (Graziani et al., 2002), CRH reduced in a concentration-dependent manner the stimulatory effect of E2 on cell growth at both time points (not shown), with the maximal inhibition being achieved at 100 nM CRH; therefore, all subsequent experiments were performed with CRH given at a fixed 100 nM concentration (Fig. 1A and B). The profile of CRH inhibition met the criteria for non-competitive antagonism, and the inhibitory effect of CRH reached statistical significance when both CRH and E2 were given at 100 nM; therefore CRH and E2 were used at these concentrations in all subsequent experiments.

The antiproliferative effect of CRH was not associated with induction of apoptosis, as indicated by staining experiments with the fluorescent nuclear dye Hoechst 33258 (Fig. 2).

In 72-h incubation experiments with the non-selective CRH antagonists’ astressin (Gulyas et al., 1995), E2 significantly increased cell proliferation compared to baseline and this effect was almost abolished by CRH. Astressin reversed in a concentration-dependent manner CRH inhibition and even tended to potentiate E2 stimulation at the higher concentration (Fig. 3A). When given alone, astressin was able to increase cell proliferation, and also tended to further increase E2 stimulation, although the latter effect did not attain statistical significance compared to E2 alone (Fig. 3B). Likewise, the selective antagonist of CRH-R1 receptors antalarmin (Webster et al., 1996) com-

![Fig. 2. Effects of CRH on MCF7 cell vitality. Cells were incubated with vehicle (control, panel A) or with medium containing 100 nM CRH (panel B) for 48 h and stained with the fluorescent nuclear dye Hoechst 33258. Figure shows a representative experiment.](image)
Fig. 4. The selective CRH-R1 receptor antagonist antalarmin reverts CRH inhibition of E2-stimulated MCF7 cell proliferation. Each symbol value represents the mean of cell counts performed in quadruplicate. Bars: S.E.M. The results are representative of one out of two repeated experiments. Symbols ‘**’ and ‘***’: \( p < 0.01 \) and \( p < 0.001 \) vs. controls, respectively; symbol ‘◦’: \( p < 0.05 \) vs. E2 alone.

pletely reversed the inhibitory effect of CRH over E2-stimulated cell proliferation (Fig. 4).

Having observed a functional, receptor-operated effect of CRH on MCF7 cell proliferation, we used the RNase protection assay to demonstrate that this cell type express the CRH-R1 receptor subtype transcript under basal conditions (Fig. 5).

Using the RT-PCR approach, we found that MCF7 cells also express mRNA encoding for the ligand, CRH, to a higher extent compared to other cell types carried out in the same experi-

Fig. 5. Expression of CRH-R1 mRNA in MCF7 cells. A representative autoradiogram of RNase protection assay of CRH-R1 mRNA is shown; total RNA isolated from MCF-7 cells was hybridized with the antisense probe specific to human CRH-R1, hCRH-R1 (5 × 10^5 cpm), and hGAPDH (8000 cpm). The protected fragments were resolved on a 5% polyacrylamide 8 M urea gel.

Fig. 6. Panel A: expression of CRH transcript in MCF-7 cells. RNA samples from MCF-7, human endometrial adenocarcinoma IK and the human B lymphoblastoid cell line MT-1 cells were subjected to RT-PCR analysis, using primers specific for CRH. Negative control (NC): MCF-7 RNA w/o reverse transcriptase. M: 1 Kb plus DNA ladder (Invitrogen). Panel B: intracellular and secreted CRH immunoreactivity measured after 72-h experiments with MCF7 cells incubated under basal conditions. Data are expressed as pg CRH/well, the means ± S.E.M. of six replicates per group.

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4. Discussion

In this study we found that CRH was able to antagonize the proliferative effects of E2 on MCF7 cell growth in vitro. According to the classical receptor theory, antagonism exerted by CRH on the effects of E2 was non-competitive, since the dose–response curves of E2 in the absence and in the presence
of the antagonist tended to diverge by increasing the dose of the agonist. Indeed, as a non-competitive antagonist, CRH does not interact with E2 receptors and acts on specific CRH binding sites. Pharmacological evidence indicated that CRH exerts its antiproliferative effects via the interaction with a CRH-R1 receptor subtype, and RNase protection analysis confirmed this assumption, since CRH-R1 receptor was found to be expressed under basal conditions in MCF7 cells. This finding is consistent with the data recently reported by Parham et al. (2004).

To our knowledge this is the first report of an antiproliferative effect of CRH on human breast cancer cells. This observation raises a question as to whether CRH might also exert antiproliferative actions on human breast cancer in vivo. On this regard, a first point of discussion concerns with the presence of CRH receptors in this type of tumor. In the largest study published so far on the topic of CRH receptors in human cancers (Reubi et al., 2003), breast cancer was not investigated, and no other report exists in the literature, if we exclude the above mentioned paper by Parham et al. (2004) who, by the way, referred to MCF7 cell lines rather than human tumor tissues and cells. Thus, the expression of CRH receptors in human breast cancer remains to be investigated.

Another relevant question concerns with the origin, whether systemic or tissue-borne, of CRH acting on the tumor. Circulating levels of CRH under basal conditions range between 2–20 pg/ml as lower limit and 30–100 pg/ml as upper limit, depending on the analytical method used (Cunnah et al., 1987; Linton et al., 1987). These values do not appear to vary in a significant manner in association to conditions such as Cush- ing or Addison disease, insulin-induced hypoglycaemia, treatment with dexamethasone or feeding; however, increases of 2 orders of magnitude were measured in pregnant women near term (Cunnah et al., 1987; Linton et al., 1987). Despite such huge increases, CRH does not significantly interfere with ACTH secretion during pregnancy, because it is for the most part bound by a CRH binding protein (CRH-BP) produced in the liver and placenta (Petraglia et al., 1993; Behan et al., 1995); such trapping mechanism by CRH-BP is thought to prevent hypothalamo-pituitary-adrenal (HPA) axis activation as well as other central and peripheral effects of systemic CRH (Behan et al., 1995). Thus, because of the negligible levels of circulating CRH (with the exception of the third trimester of pregnancy) and the sequestering action of CRH-BP, it is unlikely that systemic CRH may play a crucial role in binding and activating putative CRH receptors at the level of breast cancer tissues.

Another possibility is that CRH is produced locally within the tumor. In fact, CRH-like immunoreactivity was detected in 14 out of 40 (35%) tumor biopsy specimens obtained from breast cancer patients immunostaining was distributed in the cytoplasm or in the nucleus, and did not appear to be correlated with the histological grade or the clinical stage of tumor (Ciocca et al., 1990). Usually immunohistochemistry does not allow to distinguish between intracellular synthesis of the peptide and uptake from the extracellular space. However, in MCF7 cells we found the CRH gene is highly expressed and the peptide is actively secreted by the cells, thus providing the basis to postulate a mechanism according to which CRH is secreted by tumor cells and exerts local – paracrine or autocrine – inhibitory action over cell growth.

In conclusion, here we showed that CRH acts on CRH-R1 receptors to oppose the proliferative effects of estrogen on MCF7 cells, probably through a local paracrine–autocrine mode of action. Further studies are needed to clarify whether these effects of CRH are clinically relevant in breast cancer patients.

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[Further references listed...]

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