Relaxin Activates the L-Arginine-Nitric Oxide Pathway in Human Breast Cancer Cells

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ABSTRACT

Recently, we demonstrated that relaxin (RLX), a peptide hormone of ovarian origin, inhibits growth and promotes differentiation of MCF-7 breast adenocarcinoma cells. We also showed that RLX stimulates the production of nitric oxide (NO) in several cell types. NO has been reported to have antitumor activity by inhibiting proliferation, promoting differentiation, and reducing the metastatic spread of some tumor cell types. In this study, we aimed at evaluating whether RLX influences the L-arginine-NO pathway in MCF-7 cells. The cells were grown in the absence or presence of RLX at different concentrations, and cell proliferation, constitutive and inducible NO synthase activities, nitrite production, and intracellular levels of cyclic GMP were investigated. The results obtained indicate that RLX increases inducible NO synthase activity and potentiates NO production. This was accompanied by an elevation of intracellular cyclic GMP, which is known to mediate the cell response to NO. The RLX-induced activation of the L-arginine-NO pathway in the MCF-7 cells was inversely related to the rate of cell proliferation. These results suggest that the cytostatic effect of RLX on MCF-7 breast cancer cells may rely on its ability to stimulate endogenous production of NO.

INTRODUCTION

Previous studies from our laboratory have shown that the peptide hormone RLX stimulates the development of the mammary gland (1–6). We could also demonstrate that RLX is capable of influencing growth of human breast adenocarcinoma MCF-7 cells in culture, causing a powerful inhibition of cell proliferation at micromolar concentrations (7). Moreover, inhibition of MCF-7 cell growth by RLX was shown to be accompanied by induction of differentiation and by an increase in the expression of cell-cell adhesion molecules (8, 9). The mechanisms of action of RLX at the cellular level are poorly understood. The results of our more recent studies on mast cells, platelets, and blood vessels (10–12) indicate that RLX evokes the responses of its different targets through a common mechanism, consistent with stimulation of the endogenous production of NO. It has been shown for some years that tumor cells are susceptible to NO cytostasis (13–17). NO exerts this action through several mechanisms, including an inhibition of DNA synthesis (14, 18–21), mitochondrial respiration (15, 22, 23), and cytochrome P-450 activity (24) and an interference with iron-sulfur proteins (14, 25, 26). NO has also been considered as an important mediator of differentiation for neuroblastoma cells (27). Moreover, an inverse relationship between the generation of NO by colorectal adenocarcinoma cells and their metastatic potential has been reported (28). Interestingly, some murine breast adenocarcinoma cell lines and melanoma cell lines have been shown to express iNOS, either spontaneously or after appropriate stimulation (20, 29–31), and the consequent generation of NO has been found to inhibit their own DNA synthesis and to correlate inversely with production of metastases. The antitumor effect of NO implies that compounds that increase NO formation, such as RLX, may be useful in attenuating the growth of tumor cells. This study was designed to verify whether the antitumor effect of RLX on MCF-7 cells recognized in our previous studies may rely on the ability of the hormone to stimulate NO production.

MATERIALS AND METHODS

Materials. Porcine RLX standard, purified according to the method of Sherwood and O’Byrne (32), was the generous gift of Dr. O. D. Sherwood (University of Illinois at Urbana-Champaign, Urbana, IL). The MCF-7 human breast adenocarcinoma cell line (33) was obtained from the American Type Culture Collection (Rockville, MD; ATCC HTB22) and used between the 40th and 60th passages in culture. Modified Coon’s medium for cell culture was purchased from Unipath (Milano, Italy). FCS was from Biological Industries (Beth Haemek, Israel), and tissue culture plasticware was from Costar (Milano, Italy). Antibiotics for cell culture, trypsin solution, EGTA, EDTA, sulfinic acid, N-1-naphthylethylamine dihydrochloride, DTT, calmodulin (free base), NADPH, and nitrate reductase were from Sigma Chemical Co. (St. Louis, MO). [methyl-3H]thymidine, [3H]-arginine, and RIA kit for [3H]serum albumin (human), TCA, 1,1,2-trichloro-trifluoroethane, l-NMMA, superoxide dismutase from bovine erythrocytes, sodium nitrite, and trifluoperazine were from Sigma–Aldrich (Milano, Italy). IBMX was from Aldrich (Dorset, United Kingdom). Stock solutions of IBMX were prepared in 0.1 N NaOH and then diluted in Krebs’ buffer. l-arginine was from Ultrafine Chemicals, Ltd. (Manchester, United Kingdom).

Cell Culture. The MCF-7 cells were maintained in culture in medium supplemented with 10% FCS, added with 100 IU/ml of penicillin and 100 µg/ml of streptomycin, in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. The cells were released from the culture plates by treatment with 0.5% trypsin in PBS for 10 min and allowed to seed for 24 h before entering the experiments. At the beginning of the experiments, the medium was replaced with medium alone (controls) or medium added with RLX at concentrations ranging from 10−8 to 10−6 M. The cells were grown for 96 h before being processed for functional assays. Cell viability was assayed by the trypan blue exclusion test before and after incubation with either medium or 10−8 M RLX. The percentage of viable cells did not differ significantly in the various assays performed (89% before incubation, 97% after 96 h of medium alone, and 96% after 96 h of RLX).

Evaluation of Cell Growth. MCF-7 cell growth was determined by the [3H]thymidine incorporation assay. Briefly, 104 cells were seeded in 24-well plates and left to grow to subconfluence. After a 24-h incubation in steady-state medium (i.e., medium supplemented with 0.1% FCS), the medium was substituted with medium containing 10% FCS, added or not with RLX at the noted concentrations. At the end of the incubations, the cells were pulsed for 4 h with 0.5 µCi [methyl-3H]thymidine (specific activity, 46 Ci/mmol) per well. The medium was then removed, and DNA was precipitated with cold 3% TCA and extracted with 1 ml of 0.3 M NaOH. The recovered radioactivity was measured in a beta counter (model 1900 TR; Packard Tri-Carb, Zürich, Switzerland). The experiments were performed in quadruplicate, and the values were expressed as dpm well (mean ± SE).

Evaluation of NO Synthase Activity. NO synthase activity was determined in cell homogenates by measuring [3H]citrulline formation from [3H]-arginine, according to the method reported by Mollace et al. (34). Briefly, 106 MCF-7 cells
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were seeded in 35-mm-diameter culture dishes, allowed to grow to subconfluence, and then incubated with medium added with 10% FCS in the absence (controls) or presence of RLX at the noted concentrations and time of exposure. The cells were then washed and scraped in Dulbecco’s PBS. The samples were quickly frozen and kept at —70°C until needed. At the moment of the assay, the cells were homogenized in HEPES buffer (20 mM, pH 7.2) containing 0.32 M sucrose, 1 mM EDTA, and 1 mM DTT and then centrifuged at 39,000 x g for 30 min. Part of the MCF-7 cell homogenates was used for determination of global NOS activity. The samples (340 μl) were added with 60 μl of a medium of the following composition: 2 mM NADPH, 0.45 mM CaCl2, 10 μM calmodulin, 200 mM L-arginine, and 5 μCi/ml [3H]-L-arginine. After 60 min of incubation at 37°C, the mixture was loaded on 1 ml Dowex AG50W×8 (Na+ form) column (Sigma) and eluted by 5 ml of distilled water. The [3H]citrulline obtained from enzyme activity was measured by a beta counter (Packard), and the ratio between labeled citrulline (dpm) and milligrams of proteins, the latter determined in the homogenate according to Lowry et al. (35), was taken as NOS activity. Another part of the MCF-7 cell homogenates was used to determine the activity of Ca2+/calmodulin-independent iNOS. In this latter case, the homogenates were suspended in Ca2+/calmodulin-free buffer containing 1 mM EGTA and the calmodulin inhibitor trifluoperazine (100 μM). The activity of cNOS was determined from the difference between the values of labeled citrulline produced in the presence of Ca2+ and calmodulin and those produced in the absence of Ca2+ and calmodulin, in the presence of EGTA and trifluoperazine, as described by Salter et al. (36). The values obtained from the assays are expressed as the means (±SE) of eight independent experiments, performed in duplicate.

Evaluation of NO Production. This was performed by determination of the amount of nitrite (NO2−), a stable end-product of NO metabolism, in MCF-7 cell homogenates. The cells were seeded into 6-well plates at a 106 concentration, allowed to grow to subconfluence, and then incubated for 96 h in the absence (controls) or presence of RLX at the noted concentrations. To avoid interference by L-arginine contained in the FCS on NO production by the cells, the experiments were carried out with steady-state medium. In some cultures, the medium was added with the NOS inhibitor L-NMMA (10−4 M) 1 h before incubation of the cells with RLX or with medium alone. At the end of the experiments, the cells were detached from culture plates, centrifuged, resuspended in 500 μl of PBS, and sonicated. The amounts of NO2− were measured spectrophotometrically by the Griess reaction. Briefly, the samples were added with nitrate reductase (276 milliunits) and NADPH (40 μM) and then allowed to react with the Griess reagent (aqueous solution of 1% sulfanilamide and 0.1% naphthylethendiamine dihydrochloride in 2.5% H3PO4) to form a stable chromophore absorbing at 546 nm. The values were obtained by comparison with standard concentrations of sodium nitrite and expressed as net amounts of NO2− per mg of protein (37). The protein concentrations were determined by the Lowry method. The values reported are the means (± SE) of two independent experiments performed in quadruplicate.

Evaluation of cGMP. cGMP is known to be the mediator of the response of the cells to NO (38). To ascertain whether intracellular cGMP undergoes changes in MCF-7 cells upon RLX treatment, 106 cells were seeded into 6-well plates, allowed to grow to subconfluence, and then incubated for 96 h with medium added with 10% FCS, in the absence (controls) or presence of RLX at the noted concentrations. One h before the end of the experiments, IBMX (50 μM) was added to the cell cultures to inhibit phosphodiesterase activity. The levels of cGMP were measured in the aqueous phase of 5% TCA extracts of the cells, as described previously (39). The values are expressed as fmol of cGMP per mg of protein. The protein concentrations were determined by the Lowry method. The values reported are the means (± SE) of eight determinations from two independent experiments performed in quadruplicate.

Statistical Analysis. The data were analyzed for significance by the Student’s t test for unpaired values. P ≤ 0.05 was considered significant.
of RLX. The peptide causes a concentration-related increase in cGMP levels. Values are
seeded at a concentration of 10^6/well and incubated for 96 h in the absence or presence
of RLX. The peptide causes a concentration-related increase in cGMP levels. Values are
means; bars, SE. RLX versus control: *P < 0.001.

RESULTS

The MCF-7 cells cultured in medium added with RLX underwent a significant increase in [3H]thymidine incorporation at the lower concentrations of the peptide as compared with their control counterparts. Conversely, a significant decrease in [3H]thymidine incorporation was obtained with the higher RLX concentration (Fig. 1) in the absence of any substantial decrease in cell viability.

Determination of iNOS and cNOS activities revealed that the MCF-7 cells express spontaneously both enzymatic activities at low levels. Upon RLX treatment, a significant increase in iNOS activity was observed at every RLX concentration assayed. On the other hand, a slight but significant increase of cNOS activity was found with 10^-9 m RLX, followed by a significant depression of the enzyme activity at the higher RLX concentrations (Fig. 2).

An involvement of the L-arginine-NO-pathway in the response of the MCF-7 cells to RLX is further evidenced by the determinations of NO2^- amounts, starting from a peptide concentration of 10^-8 M onwards. As expected, addition of the NOS inhibitor L-NMMA decreased both basal and RLX-induced increases in NO2^- concentrations (Fig. 3). Treatment with RLX also caused a significant elevation of the intracellular cGMP levels (Fig. 4), in a fashion which parallels closely the rise of iNOS activity.

DISCUSSION

The results of this study show that RLX stimulates the L-arginine-NO-pathway in human breast adenocarcinoma MCF-7 cells. Upon RLX treatment, the activity of iNOS is enhanced, and this is accompanied by a rise in NO production and by an elevation of the intracellular levels of cGMP, which is known to mediate the biological effects of NO (38). These effects of RLX are maximal at concentrations ranging from 10^-8 M to 10^-6 M. Of note, the parallel experiments on MCF-7 cell proliferation, together with the results of previous investigations (7, 8), showed that RLX, at micromolar concentrations, has antimetopic properties, which have been shown not to be related to the induction of apoptosis (8). Therefore, it is conceivable that the antiproliferative effect of RLX on MCF-7 cells is exerted through an activation of the L-arginine-NO-pathway. This agrees with previous reports that NO has a powerful cytostatic action on tumor cells by inhibiting DNA synthesis (14, 18–21) and causing oxidative injury (14, 15, 22–26).

It is conceivable that the differentiation-promoting action of RLX on MCF-7 cells found in our previous studies (8, 9) may also depend on the stimulation of NO production by the peptide. In this context, it is worth noting that NO has been identified as the mediator of cytokine-induced differentiation of cultured neuroblastoma cells (27).

This study first provides evidence that MCF-7 cells express both the Ca2^+ /calmodulin-dependent and -independent NOS isoforms and that RLX acts as a stimulator of NOS activities in these cells, as it does in mast cells, platelets, and blood vessels (10–12). Of note, in the MCF-7 cells, RLX increases iNOS activity in a dose-dependent fashion, whereas it stimulates cNOS activity only at the lower, nanomolar concentration, with a significant inhibition of cNOS being observed with the higher RLX concentrations. This may be explained on the basis of previous reports that endogenously generated NO markedly inhibits cNOS by interfering with the function of the heme prosthetic group of the enzyme (40), whereas iNOS is much less susceptible to inhibition by NO (41). It is conceivable that, in the MCF-7 cells, the enhancement of iNOS activity and the consequent rise of NO production evoked by RLX at the highest concentrations may produce a depression of cNOS activity due to negative feed-back exerted by NO itself.

Based on the newly recognized property of RLX in stimulating iNOS activity and NO production by MCF-7 cells, the hypothesis can be drawn out that the peptide may also reduce the metastatic potential of breast cancer cells. This hypothesis is supported by previous reports that the levels of iNOS and of NO were higher in nonmetastatic than in metastatic cells of murine mammary adenocarcinoma and melanoma cell lines (20, 29–31) and agrees with our previous observation that RLX enhances the expression of the cell-cell adhesion molecule E-cadherin (8), which has been found to prevent tumor invasion and, hence, metastatic spread (42–47).

REFERENCES


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