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Identification, Characterization, and Biological Activity of Specific Receptors for Natural (Ghrelin) and Synthetic Growth Hormone Secretagogues and Analogs in Human Breast Carcinomas and Cell Lines*

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ABSTRACT

The family of GH secretagogues (GHS) includes synthetic peptidyl (hexarelin) and nonpeptidyl (MK-0677) molecules possessing specific receptors in the pituitary and central nervous system as well as in peripheral tissues, including the heart and some endocrine organs. A gastric-derived peptide, named ghrelin, has recently been proposed as the natural ligand of the GHS receptors (GHS-Rs). The presence of specific GHS-Rs has now been investigated in nontumoral and neoplastic human breast tissue using a radioiodinated peptidyl GHS ([^{125}I]-Tyr-Ala-hexarelin) as ligand. Specific binding sites for GHS were detected in membranes from several types of breast carcinomas, whereas a negligible binding was found in fibroadenomas and mammary parenchyma. The highest binding activity was found in well-differentiated (G1) invasive breast carcinomas and was progressively reduced in moderately (G2) to poorly (G3) differentiated tumors. [^{125}I]-Tyr-Ala-hexarelin bound to tumor membranes was displaced by different unlabeled GHS such as hexarelin, Tyr-Ala-hexarelin, human ghrelin, and MK-0677 as well as by desoctanoyl-ghrelin and hexarelin derivative EP-80317, which are devoid of GH-releasing properties *in vivo*. In contrast, no competition was seen between radiolabeled Tyr-Ala-hexarelin and some peptides (CRF and insulin-like growth factor I) structurally and functionally unrelated to hexarelin or when GHRH and SRIF were tested in the displacement studies. The presence of specific GHS binding sites was also demonstrated in three different human breast carcinoma cell lines (MCF7, T47D, and MDA-MB231), in which, surprisingly, no messenger RNA for GHS-R1a was demonstrated by RT-PCR. In these cell lines, ghrelin (as well as hexarelin, MK-0677, EP-80317, and even desoctanoyl ghrelin) caused a significant inhibition of cell proliferation at concentrations close to their binding affinity. In conclusion, this study provides the first demonstration of specific GHS binding sites, other than GHS-R1, in breast cancer. These receptors probably mediate growth inhibitory effects on breast carcinoma cells *in vitro*. (*J Clin Endocrinol Metab* **86:** 1738–1745, 2001)

GH SECRETAGOGUES (GHS) are synthetic, peptidyl (GH-releasing peptides, GHRPs) and nonpeptidyl molecules, which possess strong, dose-dependent and reproducible GH-releasing activity *in vivo*. This activity can be demonstrated in several species (including human) after iv, sc, intranasal, and even oral administration (1–3). Both peptidyl and nonpeptidyl compounds also possess significant PRL-releasing and ACTH/cortisol-releasing effects (4, 5). The neuroendocrine activities of GHS are mediated by specific receptors that have originally been identified in the pituitary and the hypothalamus in humans, as well as in rats, using radiolabeled nonpeptidyl ([^{35}S]MK-0677) or radiolabeled peptidyl GHS, such as ([^{125}I]-Tyr-Ala-hexarelin (6–9).

A specific animal and human GHS-receptor (GHS-R) has been cloned (9). It is encoded by a rare messenger RNA (mRNA) with a predicted open reading frame of 366 amino acids with a transmembrane topography typified by the G protein-coupled receptor family (4, 7, 10, 11). Recently, a gastric-derived peptide, named ghrelin, has been proposed as a natural ligand of the GHS receptor (GHS-R) (12). It has been demonstrated that ghrelin has a strong stimulatory effect on GH secretion in the rat (12) and human (13) and displaces [^{125}I]-Tyr-Ala-hexarelin from human pituitary binding sites (14).

The hypothalamus and the pituitary gland show a remarkable density of GHS-R in humans as well as in animals (4, 6, 9, 15). The existence of specific GHS binding sites in the pituitary and central nervous system probably explains endocrine and central activities of GHS (3, 4, 16). However, the GHS-R distribution is not restricted to pituitary or brain. In fact, the expression of type I GHS-R mRNA has been demonstrated in the human pancreas (17) and [^{125}I]-Tyr-Ala-hexarelin labels specific binding sites in the rat and human

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heart (18–20), as well as in a wide range of other peripheral human tissues (14, 21). In addition, GHS-Rs were also found in neoplastic tissues, including pituitary adenomas (22), neuroendocrine tumors (23), and thyroid carcinomas of follicular cell origin (in both primary tumors and cell lines) (24).

The normal (nonneoplastic) mammary gland is apparently devoid of specific GHS binding sites, as opposed to other hormonally-regulated glands (14). To our knowledge, no data exist in the literature concerning breast cancer, although it has been reported that nontumoral and neoplastic mammary gland may be regulated by GH-releasing/inhibiting hormones, because specific mRNAs for GHRH and SRIF have been demonstrated in these tissues (25–27).

Based on the foregoing, the aims of the present study were: 1) to investigate the presence of GHS-R in nontumoral mammary gland and in a series of breast carcinomas and fibroadenomas by means of a radioreceptor assay, using [^{125}I]-Tyr-Ala-hexarelin as tracer; 2) to evaluate the ability of human ghrelin (either octanoylated or desoctanoylated) as well as of other peptidyl and nonpeptidyl GHS (hexarelin, Tyr-Ala-hexarelin, and MK-0677) and analogs (EP-80317 and EP-9399) to compete with the radioligand for binding sites in the above tumors; and 3) to study the effects of these compounds on the proliferation of estrogen-dependent (MCF7, T47D) and estrogen-independent (MDA-MB231) human breast carcinoma cell lines *in vitro*.

Materials and Methods

Chemicals

Human ghrelin (Gly-Ser-Ser-(O *n*-octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg-NH_2), desoctanoyl human ghrelin, MK-0677 (N-[1(R){[1,2-dihydro-1-methanesulphonylspiro-(^{3}H-indole-3, 4′-piperidin)-1′-yl]-2-(phenyl-methoxy)-ethyl}-2-amino-2-methylpropanamide methane sulphonate], hexarelin (His-D-2Me-Trp-Ala-Trp-D-Phe-Lys-NH_2), and three structurally-related analogues of hexarelin such as Tyr-Ala-hexarelin, EP-80317 [(2S, 5S)-5-amino-1,2,3,4,6,7-hexahydro-azepino (3, 2, 1-hi)indol-4-one-2-carboxylic acid-D-2Me-Trp-D-Lys-Trp-D-Phe-Lys-NH_2)], and EP-9399 [c(Trp-D-Phe-His-2Me-Trp-Ala)] were provided by Europeptides (Argenteuil, France). Human GHRH (GHRH 1–44), SRIF 1–14, human insulin-like growth factor I (IGF-I), and human CRF were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland. Tamoxifen and 17-β estradiol were purchased from Sigma (St. Louis, MO). [^{125}I]-Tyr-Ala-hexarelin (SA 2000 Ci/mmol) was iodinated using a lactoperoxidase method and purified by reverse-phase high-performance liquid chromatography, as previously described (6, 7). [^{3}H]-thymidine (SA 2000 Ci/mmol) was purchased from Amersham Pharmacia Biotech Italia, (Milan, Italy). Penicillin, streptomycin, FCS, trypsin/EDTA solution, and other tissue culture reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD).

Tissue samples

Six normal breast parenchymas (obtained from mammoplasty specimens), 4 breast fibroadenomas and 24 breast carcinomas [17 invasive ductal carcinomas, 2 tubular carcinomas, 5 invasive lobular carcinomas; G1 (n = 8), G2 (n = 8), G3 (n = 8), following World Health Organization grading] were collected from surgical specimens received in the Department of Pathology of the University of Turin in years 1997–1999. No patient was undergoing hormonal (antiestrogenic) treatment. The median age of patients was 62 yr. All patients gave their informed consent for the research use on their tissues, and the study project was approved by our hospital ethical committee. A tissue fragment adjacent to one used for histopathological diagnosis was immediately frozen at −80 C and stored for 4–36 months until processed for membrane preparation and binding studies.

The receptor status (estrogen receptors, ER and progesterone receptors, PgR) and the proliferative index of all breast carcinomas have been evaluated by immunohistochemistry. The following antibodies were used: ER (1D5, diluted 1:100; DAKO Corp., Glostrup, Denmark), PgR (diluted 1:15; BioGenex Laboratories, Inc., San Ramon, CA), ki67 (MIB-1, diluted 1:10; Immunotech, Marseille, France).

Cell cultures

Three immortalized cell lines (MCF7, T47D, and MDA-MB231), derived from human breast carcinomas, were purchased from ATCC (Rockville, MD). Two of them (MCF7 and T47D) were estrogen-dependent, whereas MDA-MB231 was an estrogen-independent cell line. All the cell lines were grown as monolayer in RPMI-1640 medium supplemented with FCS 10% and penicillin/streptomycin in a 5% CO_2-humidified atmosphere at 37 C and used in binding and cell proliferation studies.

Binding studies

GHS binding sites were assayed on membranes (30,000 × *g* pellet) isolated from human tissues or cell lines, as previously described (6), using [^{125}I]-Tyr-Ala-hexarelin as ligand. This hexarelin analog has been reported to have the same GH-releasing potency of hexarelin in rats (7) and humans (28) and to be a reliable probe for labeling human GHS-R *in vitro* (6, 7, 14, 24). In preliminary experiments, it was found that equilibrium conditions for the breast carcinomas and cell lines were similar to those found for binding to human hypothalamus and pituitary gland (6). For single-point binding assay, tissue membranes (corresponding to 100 μg membrane protein, measured using the method of Lowry *et al.*) (29) were incubated in triplicate, at 0 C for 60 min, with approximately 5 nmol/L [^{125}I]-Tyr-Ala-hexarelin in a final vol of 0.5 mL assay buffer (50 mmol/L Tris, 2 mmol/L EGTA, 0.1% BSA, 0.03% bacitracin, titrated with HCl to pH 7.3). Parallel incubations, where 2.5 μmol/L unlabeled Tyr-Ala-hexarelin was also present, were used to determine nonspecific binding, which was subtracted from total binding to yield specific binding values. The binding reaction was terminated by adding ice-cold assay buffer followed by filtration over Whatman GF/B filters. Filters were rinsed three times with assay buffer, and the radioactivity bound to membranes was measured by a Packard auto-γ counter. Specific binding was calculated as the difference between binding in the absence and in the presence of excess unlabeled Tyr-Ala-hexarelin and was expressed as a percentage of the total radioactivity added. Precautions were taken to minimize variations in the binding of [^{125}I]-Tyr-Ala-hexarelin to tissue membranes. Thus, all binding studies related to one membrane preparation were carried out using the same batch of radiotracer. To establish binding site specificity, increasing concentrations of various competitors were tested in displacement assays with [^{125}I]-Tyr-Ala-hexarelin. The concentration of a competitor agent, causing 50% inhibition of specific radioligand binding (IC_{50} value), was derived from the iterative curve-fitting Prism 3 program (GraphPad Software, Inc., San Diego, CA). In some assays, receptor binding saturation studies were also conducted by incubating tissue membranes with increasing concentrations (from 0.15–20 nmol/L) of radioligand in the absence and in the presence of a fixed amount (2.5 μmol/L) of unlabeled Tyr-Ala-hexarelin. Saturation isotherms were transformed using the method of Scatchard (30), and the dissociation constant (K_d) and number of binding sites [maximal binding capacities (B_{max})] were calculated with the GraphPad Software, Inc. Prism 3 program.

RT-PCR for GHS-R1a

RNA from the MCF7, T47D, and MDA-MB 231 cell lines was extracted by Rnazol (Roche Molecular Biochemicals, Mannheim, Germany) as described by the manufacturer. Then 3 μg RNA was retrotranscribed by Superscript Reverse Transcriptase (Life Technologies, Inc., Roskilde, Denmark) and amplified by AmpliTaq Gold Polymerase (Perkin-Elmer Corp., Foster City, CA). Primers, designed with Primer Express (Perkin-Elmer Corp.), were: 5′-CTCTGCATGCCCCTGGACCTCGTTCGC-3′ (forward) and 5′-CTGCCGATGAGACTGTAGAGGACCGTGAGAC-3′, which amplifies a 58-bp fragment of GHS-R1a. PCR was carried out by 10 min at 95 C and then by 40 cycles of 15 sec at 95 C and 1 min at

60 C. Amplified DNA was run on 2% agarose electrophoresis, and the image was acquired by Chemidoc (Bio-Rad Laboratories, Inc., Hercules, CA).

Cell proliferation studies

Cell proliferation was evaluated either by [^{3}H]-thymidine incorporation into DNA or counting cell number after appropriate incubation with different compounds. [^{3}H]-thymidine incorporation studies were performed as previously described (31). Briefly, starved breast carcinoma cell lines (2×10^5 cells/mL) were incubated at 37 C, with or without 10% FCS or estradiol (10 nmol/L), in the absence or in the presence of different concentrations (from 1 nmol/L–2 μmol/L) of GHRH, ghrelin, desoctanoyl ghrelin, MK-0677, tamoxifen, and hexarelin and its analogs (EP-80317, EP-9399). After incubation for 20 h, time needed to obtain the maximal effect of all compounds, 1 μCi/well of [^{3}H]-thymidine was added, and the incubation was continued for an additional 4 h. The reaction was then halted, and the cells were harvested onto glass-fiber filter strips. Incorporation of [^{3}H]-thymidine was measured in a scintillation counter. For cell growth studies, breast carcinoma cell lines (8×10^3 cells per mL) were seeded out in a 24-well plate containing RPMI medium supplemented with 10% FCS and grown for 96 h in the absence or in the presence of 1 μmol/L GHRH, ghrelin, desoctanoyl ghrelin, tamoxifen, and hexarelin or its analog EP-80317, with media changed every 48 h. In selected experiments, cells were synchronized, 8 h after plating, by a 36-h rest in 0.5% FCS. Cells were detached with trypsin-EDTA solution and counted in double-blind fashion, by two independent investigators, using a hemocytometer. All experiments were done in triplicate.

Statistical analysis

Values are expressed as median and range unless otherwise noted. In saturation and competition binding experiments, as well as in cell proliferation studies, they are expressed as mean ± SEM unless otherwise specified. The number of cases is indicated by *n*. Significant differences between groups were assessed by Kruskal-Wallis test. $P < 0.05$ was chosen as the level of significance.

Results

Binding of [^{125}I]-Tyr-Ala-hexarelin to membranes from nontumoral and neoplastic human breast tissue and mammary carcinoma cells

Considerable Tyr-Ala-hexarelin specific binding values were found in all breast carcinomas (Table 1). The highest specific binding activity was observed in well-differentiated (G1) invasive carcinomas, where it represented about 60–75% of total radioactivity bound. Tyr-Ala-hexarelin binding was recorded in all specimens, with binding values that were greater than those previously found (11.3–14.0%) in a classical GHS target tissue, such as the human pituitary gland (6). Specific binding was also present in moderately (G2) and poorly differentiated (G3) ductal carcinomas, with values that were about 30% and 55% lower than those detected in G1 carcinomas respectively. Tumor histotype, stage, receptor status, proliferative index, and hormonal status of patients (either pre- or postmenopausal) were not correlated to the amount of binding.

A well-detectable specific binding of [^{125}I]-Tyr-Ala-hexarelin was also present in three immortalized cell lines derived from human breast carcinomas. Tyr-Ala-hexarelin binding was most pronounced in the estrogen-dependent MCF7 cell line and in the estrogen-independent MDA-MB231 cells, with values that were comparable with those found in the pituitary gland, whereas the estrogen-dependent T47D cells showed specific binding values that were significantly lower than those detected in the above cell lines.

TABLE 1. Distribution of [^{125}I]Tyr-Ala-hexarelin binding to membranes of nontumoral mammary gland and human mammary tumors and cell lines

Histological type	Specific binding of [^{125}I]Tyr-Ala-hexarelin (% radioactivity added/ 0.1 mg protein)
Nontumoral mammary gland	
Parenchyma ($n = 6$)	0.6 (0.2–0.8)
Benign mammary tumors	
Fibroadenomas ($n = 4$)	0.8 (0.5–1.0)
Malignant mammary tumors	
Well-differentiated (G1) carcinomas ($n = 8$)	17.0 (9–53)[a,b]
Moderately differentiated (G2) carcinomas ($n = 8$)	11.5 (0.5–32)[a]
Poorly differentiated (G3) carcinomas ($n = 8$)	7.5 (3–16)[a]
Mammary carcinoma cell lines	
MCF7 ($n = 4$)	11.3 (9.5–12.5)[c]
MDA-MB-231 ($n = 4$)	10.0 (8.3–11.0)
T47D ($n = 4$)	7.3 (6.5–8.4)

Data are expressed as median (range).
[a] $P < 0.001$ *vs.* nontumoral mammary gland and benign mammary tumors.
[b] $P < 0.05$ *vs.* G3 carcinomas.
[c] $P < 0.05$ *vs.* T47D cells.

In contrast, scanty Tyr-Ala-hexarelin binding was observed in the nontumoral breast parenchyma and in all the fibroadenomas studied (Table 1).

Specificity of binding and saturation studies

To determine whether the binding of [^{125}I]-Tyr-Ala-hexarelin to tissue membranes shows the properties typical of ligand-receptor interaction, the binding of radiotracer was investigated in more detail in some specimens of mammary carcinomas that yielded sufficient amounts of membranes for these studies and in the MCF7 cells in which the presence of a specific [^{125}I]-Tyr-Ala-hexarelin binding was also demonstrated. The specificity of [^{125}I]-Tyr-Ala-hexarelin binding to membranes from G1 mammary carcinomas and MCF7 cells was established by competitive binding experiments, using several natural compounds that stimulate (human ghrelin, GHRH) or inhibit (SRIF 14) GH secretion, and some synthetic peptidyl (hexarelin, Tyr-Ala-hexarelin) and nonpeptidyl (MK-0677) GHS. Various structurally-related analogs of hexarelin (EP-80317 and EP-9399) and ghrelin (desoctanoyl ghrelin), which do not have GH-releasing activity *in vivo* (Refs. 32 and 33; and Locatelli V., T. Reissmann, I. C. Robinson, personal communications), and some hormones (CRF and IGF-I) functionally unrelated to hexarelin were also studied in these competitive binding experiments. The binding of [^{125}I]-Tyr-Ala-hexarelin to membranes of G1 carcinomas was completely displaced by increasing concentrations of unlabeled hexarelin, Tyr-Ala-hexarelin, EP-80317, MK-0677, ghrelin, and desoctanoyl ghrelin, whereas none of the structurally and functionally unrelated peptides (CRF and IGF-I), as well as GHRH, SRIF-14, and EP-9399 inhibited the binding of radiotracer. Hexarelin, Tyr-Ala-hexarelin, and EP-80317 exhibited equally high affinity for the binding sites, whereas

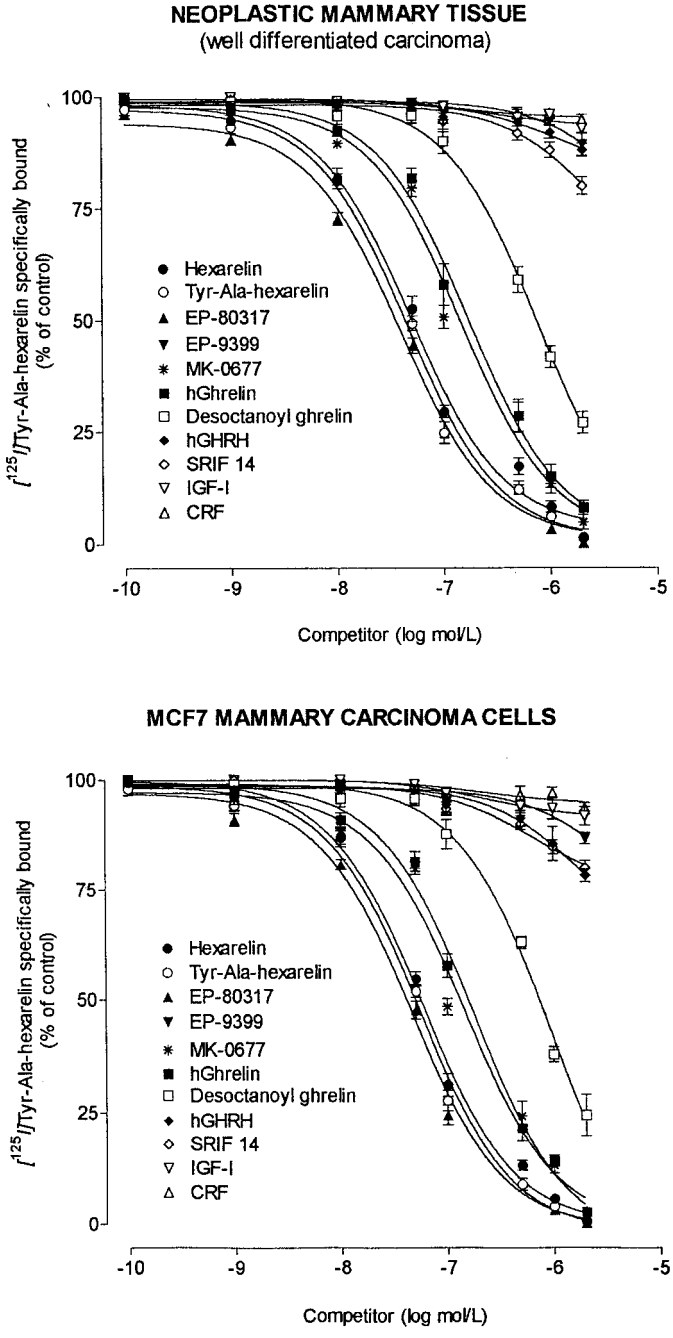

FIG. 1. Displacement of [^{125}I]-Tyr-Ala-hexarelin from membranes of a well-differentiated mammary carcinoma and MCF7 mammary carcinoma cells by different unlabeled competitors. Binding assays were conducted as described in *Materials and Methods*. The *ordinate* represents binding as a percentage of control (specific binding in the absence of unlabeled competitor). Values are mean ± SEM of four separate experiments. hGhrelin, Human ghrelin; hGHRH, human GHRH.

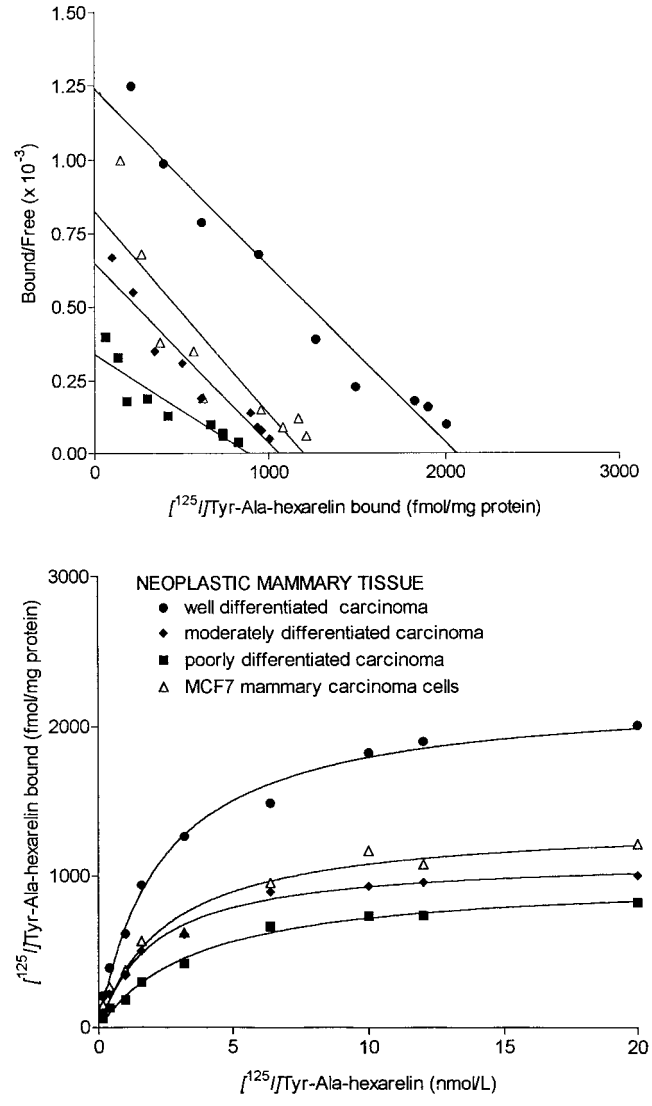

FIG. 2. Saturation of [^{125}I]-Tyr-Ala-hexarelin binding to membranes of MCF7 mammary carcinoma cells and well-, moderately, and poorly differentiated breast carcinomas. Experiments were performed by incubating a fixed amount of membrane protein (100 μg/tube) with increasing concentrations of radiolabeled Tyr-Ala-hexarelin alone (total binding) or plus 2.5 μmol/L unlabeled Tyr-Ala-hexarelin to define nonspecific binding. Specific binding values were obtained by subtracting nonspecific binding from total binding. The saturation curves of specific binding were analyzed by Scatchard analysis (*upper panel*) to calculate the B_{max} and K_d values.

MK-0677 and ghrelin, which showed a similar efficacy to each other, were less effective (3–4 times) than hexarelin and more potent (4–5 times) than desoctanoyl ghrelin in displacing [^{125}I]-Tyr-Ala-hexarelin (Fig. 1). The IC_{50} values (mean ± SEM of four separate experiments), all expressed as mol/L × 10^{-8}, were as follows: hexarelin, 5.3 ± 0.4; Tyr-Ala-hexarelin, 4.3 ± 0.3; EP-80317, 3.7 ± 0. 4; MK-0677, 15 ± 1; ghrelin, 19 ± 2; and only 80 ± 4 for desoctanoyl ghrelin. The pattern of displacement specificities in the MCF7 cells resembled that of the tumor tissue (Fig. 1).

Experiments using increasing concentrations of radioiodinated Tyr-Ala-hexarelin, ranging from 0.15–20 nmol/L, provided evidence of a saturable specific binding in well- (G1), moderately (G2) and poorly (G3) differentiated breast carcinomas, as well as in MCF7 mammary carcinoma cells (Fig. 2). Scatchard analysis of these data (Fig. 2, *upper panel*) demonstrated the existence of a single class of high-affinity sites in both breast carcinomas and MCF7 cells (K_d values were: 1.8 × 10^{-9} mol/L for the well-differentiated carcinomas (G1), 1.7 × 10^{-9} mol/L for the moderately differentiated

carcinomas (G2), 2.6×10^{-9} mol/L for the poorly differentiated carcinomas (G3), and 1.4×10^{-9} mol/L for the MCF7 cells), with limited binding capacity (B_{max} values were: 2062 fmol/mg protein for the G1 carcinoma, 1057 fmol/mg protein for the G2 carcinoma, and 882 fmol/mg protein for the G3 carcinoma). The calculated B_{max} values of Tyr-Ala-hexarelin binding sites in three G1 carcinomas were significantly greater (mean ± SEM of 2198 ± 152 fmol/mg protein) than those measured in less differentiated neoplasms (1069 ± 99 fmol/mg protein for G2 and 887 ± 131 fmol/mg protein for G3 carcinomas). However, the K_d values of these three types of tumors were not substantially different from one another, being $(1.8 \pm 0.21) \times 10^{-9}$ mol/L in the G1 carcinomas, $(1.7 \pm 0.11) \times 10^{-9}$ mol/L in the G2 carcinomas, and $(2.0 \pm 0.26) \times 10^{-9}$ mol/L in the G3 carcinomas.

RT-PCR for GHS-R1a

The data presented suggest that a common receptor for ghrelin and for synthetic GHS may be expressed in mammary carcinomas. Because either ghrelin and synthetic GHS, peptidic and not peptidic, bind to GHS-R1a, we have investigated its expression in three different breast carcinoma cell lines (T47D, MCF7, and MDA-MB231). However, after RT-PCR (40 cycles), no complementary DNA corresponding to GHS-R1a was obtained from RNA extracted from mammary cell lines, whereas a significant amount of complementary DNA was amplified from the same amount of total RNA extracted from human hypothalamus and peripheral blood lymphocytes (Fig. 3). These data suggest that the high-affinity binding sites recognized by hexarelin in these cells may depend on the expression of a different receptor, which still awaits to be identified.

Biological activity of GHS-Rs in human breast cancer cells

Based on the evidence of specific GHS binding sites in both primary breast carcinomas and in the three different breast carcinoma cells (T47D, MCF7, and MDA-MB231) and on the displacement data, we investigated the effects of human ghrelin, desoctanoyl ghrelin, MK-0677, GHRH, and hexarelin and its analogues (EP-80317, EP-9399) on the cell proliferation *in vitro*. Treatments with tamoxifen, an antiestrogen that is a first-line drug in the treatment of estrogen-dependent breast cancer (34), were also included in this study as negative controls. Both the basal [^{3}H]-thymidine incorporation (serum-free conditions) and that stimulated by FCS or estradiol were studied in the estrogen-dependent MCF7 breast cancer cells. Figure 4 shows that hexarelin, EP-80317, MK-0677, tamoxifen, ghrelin, and desoctanoyl ghrelin inhibited the serum-stimulated [^{3}H]-thymidine incorporation in a concentration-dependent manner and that hexarelin, EP-80317, MK-0677, and ghrelin were more effective than tamoxifen, whereas desoctanoyl ghrelin was less effective. The calculated 50% effective doses (mean ± SEM of four separate experiments), all expressed as mol/L $\times 10^{-8}$, were as follows: hexarelin, 4.1 ± 0.8; EP-80317, 3.3 ± 1.0; MK-0677, 14 ± 2.0; ghrelin, 16 ± 1. 3; tamoxifen, 26 ± 2.1; and only 81 ± 5.7 for desoctanoyl ghrelin. In contrast, no inhibition was observed in the presence of some peptides that do not bind to GHS-R, such as GHRH and EP-9399. In addition, no change in thymidine incorporation was observed when the different compounds were incubated with MCF7 cells growing in serum-free conditions.

When hexarelin, EP-80317, MK-0677, ghrelin, desoctanoyl ghrelin, and tamoxifen were tested on the estradiol-induced

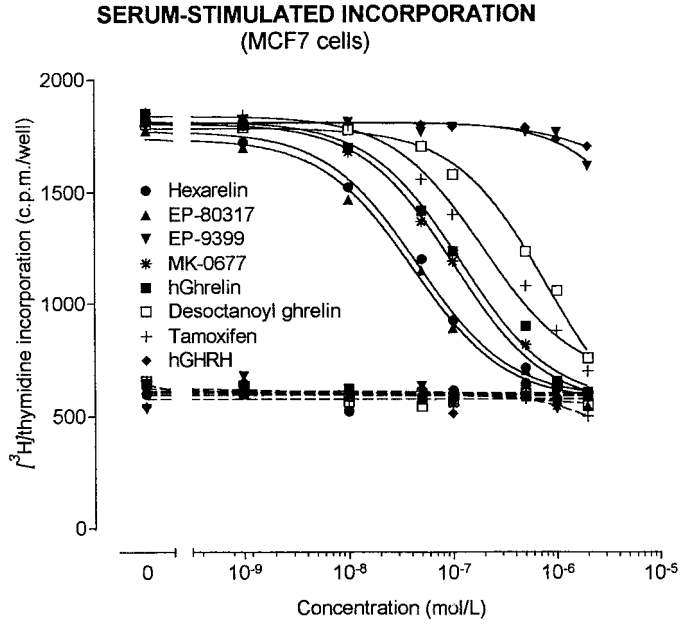

FIG. 4. Effect of human ghrelin, desoctanoyl ghrelin, MK-0677, GHRH, tamoxifen, and hexarelin and its analogs (EP-9399, EP-80317) on basal (*dashed line*) and serum-stimulated (*solid line*) incorporation of [^{3}H]thymidine into DNA by MCF7 mammary carcinoma cells. DNA synthesis was estimated by incorporation of [^{3}H]-thymidine after a 20-h incubation, with or without 10% FCS, in the absence or in the presence of different concentrations of the indicated compounds. Data are the average of duplicate assay determinants, and similar results were obtained in at least two other independent measurements.

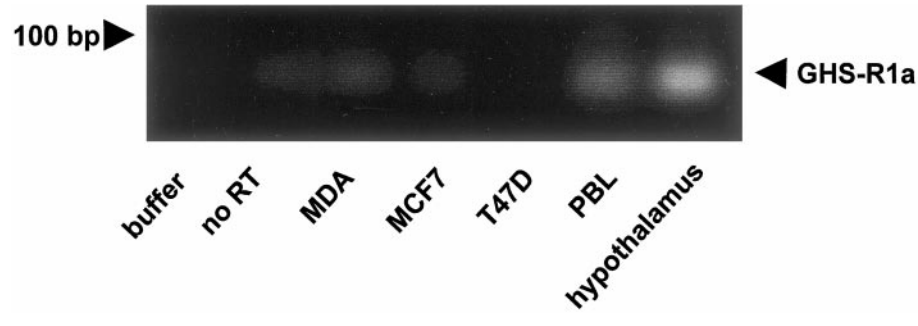

FIG. 3. Expression of GHS-R1a, by RT-PCR, in breast carcinoma cell lines. RNA was extracted from the indicated cells or tissues, retrotranscribed, and amplified as described in *Materials and Methods*. Hypothalamus and peripheral blood lymphocytes (PBL) have been used as positive controls. As negative controls, PCR reaction was carried out in the presence of RNA before retrotranscription (no RT) and in the presence of buffer alone (buffer).

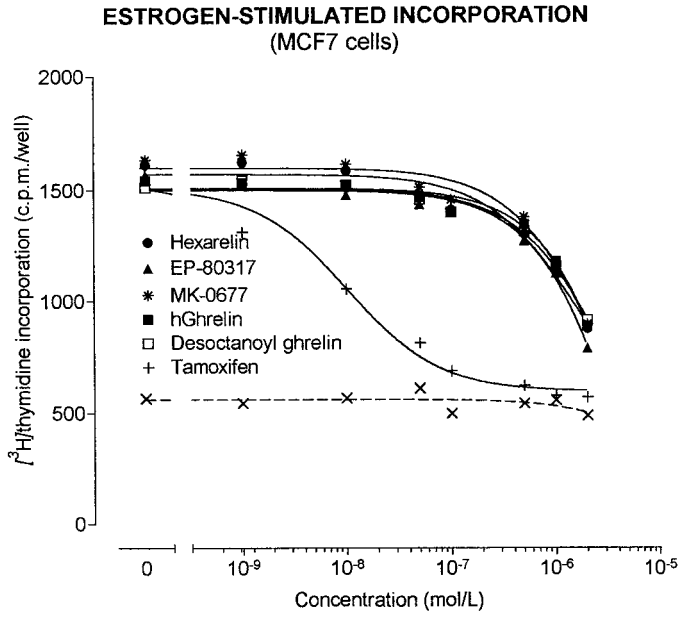

FIG. 5. Effect of human ghrelin, desoctanoyl ghrelin, MK-0677, tamoxifen, hexarelin, and EP-80317 on estradiol-stimulated incorporation of [^{3}H]-thymidine into DNA by MCF7 mammary carcinoma cells. DNA synthesis was estimated by incorporation of [^{3}H]-thymidine after a 20-h incubation, with or without 10 nmol/L estradiol, in the absence or in the presence of different concentrations of the indicated compounds. Data are the average of duplicate assay determinants, and similar results were obtained in at least two other independent measurements. The *dotted line* represents the basal value of thymidine incorporation.

[^{3}H]-thymidine incorporation (Fig 5), a clear dose-related decrease of thymidine incorporation was seen only in the presence of tamoxifen (IC_{50}: $1.0 \pm 0.2 \times 10^{-8}$ mol/L, n = 4), whereas the other substances studied showed an inhibitory effect only at highest concentrations (1–2 μmol/L).

Experiments on cell growth revealed that hexarelin, EP-80317, ghrelin, MK-0677, and tamoxifen (but not GHRH) also induced a significant inhibition of cell proliferation in MCF7 cells, at a concentration of 1×10^{-6} mol/L. The antiproliferative effect was similar for all the substances used. Since the earliest time of treatment (48 h), the compounds determined a significant decrease in cell number (ranging from 30–60%), compared with controls (Fig. 6). The simultaneous treatment with ghrelin and hexarelin did not produce a synergistic inhibition of cell growth, because the cell number remained unmodified, compared with the treatment with individual peptides. Similar results were also found on another estrogen-dependent breast cancer cell line, T47D (data not shown). When the above compounds were incubated with an estrogen-receptor negative breast cancer cell line (MDA-MB231), a significant decrease of cell growth was observed in the presence of hexarelin, EP-80317, MK-0677, ghrelin, and desoctanoyl ghrelin, whereas GHRH or tamoxifen were not effective.

Discussion

In the present study, we have demonstrated that specific receptors that bind natural (ghrelin) and synthetic peptidyl (hexarelin) and nonpeptidyl (MK-0677) GH secretagogues

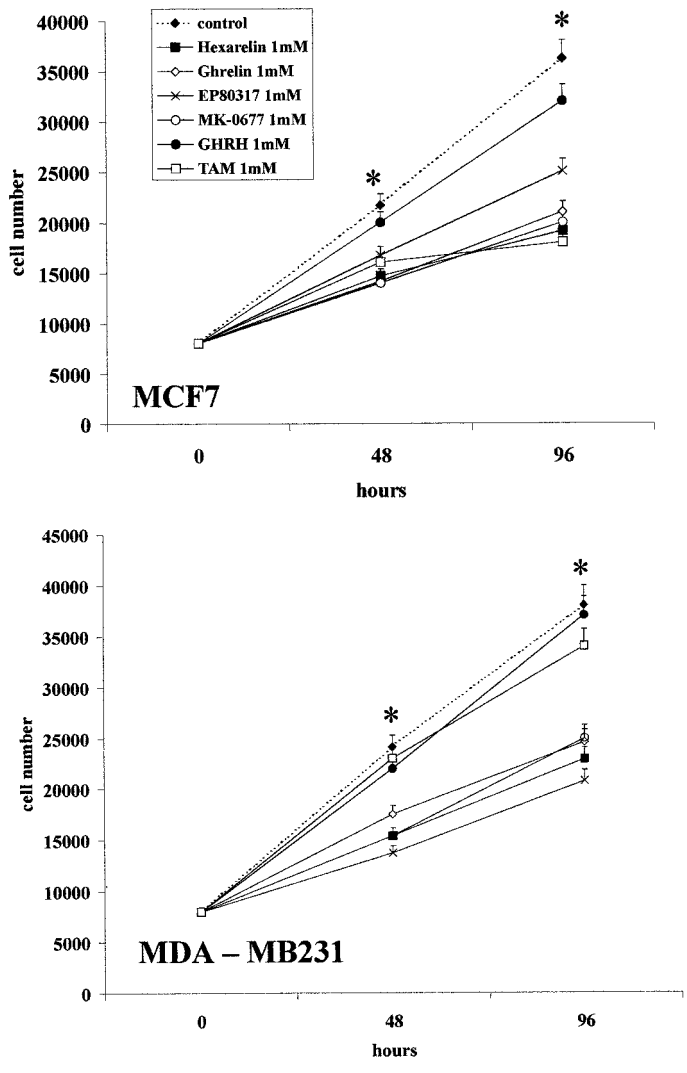

FIG. 6. Effect of 1 μmol/L ghrelin, MK-0677, GHRH, tamoxifen (TAM), and hexarelin and its analog EP-80317 on the cell proliferation of MCF7 and MDA-MB231 human breast carcinoma cell lines. Cells were grown for 96 h, in the absence or in the presence of the indicated compounds, and counted every 48 h. Values are mean ± SD of three separate experiments. *, $P > 0.01$ (ghrelin, MK-0677, tamoxifen, hexarelin, EP-80317 *vs.* control in MCF7 cells.); *, $P > 0.01$ (ghrelin, MK-0677, hexarelin, EP-80317 *vs.* control in MDA-MB231 cells). GHRH was ineffective on cell proliferation of both cell lines; tamoxifen was ineffective on MDA-MB231 cells only.

(GHS) are present in human breast carcinomas and in estrogen-dependent (MCF7 and T47D) and -independent (MDA-MB231) breast cancer cell lines. Our present study has also shown that these substances inhibit breast cancer cell proliferation *in vitro*, probably *via* activation of specific binding sites different from the classical GHS-R1.

In a previous study on the GHS-receptor (GHS-R) distribution in peripheral human tissues, we reported that GHS binding sites are not present in the nontumoral mammary gland (14, 21). We have here confirmed these previous observations, because the binding to [^{125}I]-Tyr-Ala-hexarelin was found to be undetectable in the normal breast parenchyma as well as in benign lesions (fibroadenomas). On the contrary, when breast carcinomas were studied, a specific

binding for GHS was observed. The entity of the binding was independent from the tumor histological type, stage, ER status, proliferative index, and pre- or postmenopausal age of the patients, but it was directly related to the grade of tumor differentiation. In fact, well-differentiated carcinomas showed a higher GHS binding than moderately and poorly differentiated carcinomas.

An identical profile of GHS binding had been observed in thyroid carcinomas of follicular origin (24). In these tumor tissues, the [^{125}I]-Tyr-Ala-hexarelin binding was reduced as examination moved from well-differentiated carcinomas (*i.e.* papillary carcinoma) toward the less-differentiated forms (such as poorly differentiated and anaplastic carcinomas) (24). It could therefore be suggested that functionality of (probably overexpressed) GHS binding sites is maintained in better-differentiated tumors (of both thyroid and breast origin) and is decreased in less-differentiated neoplasm.

In the present study in the neoplastic breast tissue, [^{125}I]-Tyr-Ala-hexarelin binding showed properties typical of the ligand-receptor interaction, such as high affinity, saturability, and specificity. The binding of radioligand to membranes of these tissues was inhibited by ghrelin (both octanoylated and desoctanoylated) and various peptidyl and nonpeptidyl GHS. It will be noted that the binding was inhibited even by molecules like desoctanoylated ghrelin and the peptidyl GHS EP-80317, which are devoid of any GH-releasing activity *in vivo* (32, 33). On the other hand, the binding was unaffected by a number of other peptides (GHRH, SRIF-14, IGF1, and CRF), which are structurally unrelated to peptidyl GHS. These binding properties of breast neoplastic tissue overlap with those reported in endocrine tissues, including pituitary gland, ovary, adrenal, thyroid, and testis (6, 14, 24). However, they are at variance with those generally found in other peripheral nonendocrine tissues that are target for peptidyl GHS (for instance, the heart). In fact, in the latter organs, the specific [^{125}I]-Tyr-Ala-hexarelin binding is weakly inhibited by ghrelin (14)as well as by nonpeptidyl GHS (14, 19, 35). Taken together, these data support the hypothesis that different GHS binding site subtypes may exist in peripheral organs, possibly depending on their endocrine or nonendocrine nature (9, 10, 20) but also on their normal or neoplastic nature (present study).

Specific binding for GHS, showing the same properties recorded in neoplastic breast tissue, was demonstrated also in three human breast carcinoma cell lines either estrogen-dependent (MCF7 and T47D) or -independent (MDA-MB231). In all these cell lines, GHS-R1a mRNA was not detected by RT-PCR. This implies that the specific GHS binding sites in these tissues are different from the classical GHS-R1, as well as from the specific binding sites characterized in the cardiovascular system (14, 18, 19, 35). Theoretically, the binding sites we have now described in the neoplastic breast tissue could be the same ones we have previously demonstrated in the normal and tumoral human thyroid tissue (24).

In agreement with this hypothesis, in the present study, GHS were found able to inhibit proliferation of breast carcinoma cells, as previously reported for thyroid carcinoma cell lines (24). Both octanoylated and desoctanoylated human ghrelin, as well as peptidyl and nonpeptidyl GHS, were able to inhibit serum-stimulated cell growth and thymidine incorporation at concentrations close to their binding affinity. Again notice that thymidine incorporation and cell proliferation were inhibited even by molecules like desoctanoylated ghrelin and the hexarelin derivative EP-80317, which are devoid of any GH-releasing activity *in vivo* (32, 33).

Interestingly, GHS were even found to be able to counteract the estradiol-stimulated thymidine incorporation, though high concentrations were required to obtain this effect. Moreover, in estrogen-independent MDA-MB231 cells, GHS and analogs are able to inhibit cell proliferation, whereas tamoxifen is ineffective.

In conclusion, this study shows that natural and synthetic GHS possess biological activities that are independent of their well-recognized GH-releasing activity. Thus, inhibition of breast cancer cell growth was induced even by desoctanoylated ghrelin and EP-80317, which possess no stimulatory effect on GH secretion (32, 33). The availability of molecules able to counteract tumor cell growth without any stimulatory effect on GH and PRL release could, theoretically, have potential clinical applications. In progress are experiments aimed at evaluating, *in vivo*, the antiproliferative effect of the different GHS and analogs in both estrogen-dependent and -independent tumors, mainly focusing on hexarelin derivatives devoid of GH-releasing properties, and at clarifying the intracellular mechanisms involved.

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