Specific binding sites for synthetic growth hormone secretagogues in non-tumoral and neoplastic human thyroid tissue

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Abstract

The presence of specific receptors for synthetic growth hormone secretagogues (GHSs) has been investigated in non-tumoral and neoplastic human thyroid tissue using a radio-iodinated peptidyl GHS ([125I]-labelled Tyr-Ala-hexarelin) as ligand. Specific binding sites for Tyr-Ala-hexarelin were detected in membranes from non-tumoral and follicular-derived neoplastic thyroid tissue, but not in thyroid tumours (medullary carcinomas) of parafollicular (C cell) origin. The binding activity was greatest in thyroid tumours (medullary carcinomas), followed by poorly differentiated neoplasms (papillary and follicular carcinomas) of parafollicular (C cell) origin. The binding activity was greatest in well differentiated neoplasms (papillary and follicular carcinomas), followed by poorly differentiated carcinomas, non-tumoral thyroid parenchyma, follicular adenomas and anaplastic carcinomas. Both peptidyl (Tyr-Ala-hexarelin, hexarelin, growth hormone releasing peptide (GHRP6) and non-peptidyl (MK 0677) GHSs completely displaced the radioligand from binding sites of non-tumoral thyroid gland, but MK 0677 was significantly less potent. The IC50 values were (1.9 ± 0.3) × 10−8 mol/l for Tyr-Ala-hexarelin, (2 ± 0.2) × 10−8 mol/l for hexarelin, (2.4 ± 0.3) × 10−8 mol/l for GHRP6 and only (1.5 ± 0.4) × 10−7 mol/l for MK 0677. Similar IC50 values were found in neoplastic thyroid tissue. Scatchard analysis of the binding revealed a finite number of binding sites in non-tumoral (Bmax: 1232 ± 32 fmol/mg protein, n=3) and neoplastic (papillary carcinomas) thyroid tissue (Bmax: 2483 ± 380 fmol/mg protein, n=5), with dissociation constants (Kd) of (3.8 ± 0.3) × 10−9 and (4.4 ± 0.6) × 10−9 mol/l, respectively. On the basis of this evidence, we investigated the effects of some GHS on the proliferation of three different human follicular thyroid carcinoma cell lines (NPA, WRO and ARO) in which the presence of specific GHS receptors was also demonstrated. Tyr-Ala-hexarelin, GHRP6 and MK 0677 were able to inhibit serum-stimulated [3H]thymidine incorporation in NPA cells at concentrations close to their binding affinity. These substances also caused a significant inhibition of cell proliferation, which was evident at the earliest time of treatment (24 h) in all the cell lines, and at the latest time (96 h) in NPA cells only.

In conclusion, this paper confirms the existence of specific binding sites for GHS in normal thyroid tissue and demonstrates, for the first time, that these binding sites are present in papillary and follicular carcinomas, low in anaplastic carcinomas and absent in medullary carcinomas of the thyroid. This work also provides evidence of a growth-inhibitory effect of GHS on cell lines derived from follicular thyroid cancers.

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Introduction

The family of synthetic growth hormone secretagogues (GHSs) includes peptidyl molecules such as growth hormone releasing peptides (GHRP) 1, 2 and 6, hexarelin, Tyr-Ala-hexarelin, ipamorelin and EP 51216, and non-peptidyl compounds having benzolactamic (L-692,585) and spiroindolinic (MK 0677) structures (Camanni et al. 1998, Deghenghi 1998, Casanueva & Dieguez 1999). Interestingly, GHS possess other endocrine activities such as prolactin-, adrenocorticotropic- and cortisol-releasing effects, and non-endocrine activities such as control of sleep, food intake and cardiotropic actions (Ghigo et al. 1997, Smith et al. 1997, Bowers 1998, Camanni et al. 1998, Casanueva & Dieguez 1999, Muccioli et al. 1999). Recent evidence indicates that the neuroendocrine effects of GHS are mediated by the activation of specific receptors present in the hypothalamo–pituitary system (Pong et al. 1996, Muccioli et al. 1998, Ong et al. 1998b). Specific GHS receptor subtypes (types Ia and Ib), have been cloned (Howard et al. 1996). They are encoded by a rare mRNA having a predicted open reading frame of 366 amino acids
with a transmembrane topography typified by the G-protein coupled receptor (GPR) family. The receptor sequence does not show significant homology with other receptors known to date, and receptor transcripts are expressed mainly in the pituitary and hypothalamus, but also in other brain areas (Howard et al. 1996, Guan et al. 1997, Smith et al. 1997). Moreover, other receptors having partial homology with both GHS and neuretinin receptors have recently been characterised and named GPR 38, GPR 39 and FM-3 (McKee et al. 1997, Tan et al. 1998). These orphan receptors also bind GHS and are expressed mainly in peripheral tissues such as thyroid, stomach, intestine, bone marrow and spleen (McKee et al. 1997).

These findings are in agreement with previous studies showing specific peptidyl GHS binding sites in peripheral endocrine and non-endocrine tissues, including the thyroid (Muccioli et al. 1997b, 1999, Ong et al. 1998a, Deghenghi et al. 1999).

On the basis of the foregoing, the aim of the present study was to investigate further the presence of specific GHS binding in non-tumoral thyroid tissue, and in human neoplasms of follicular and parafollicular (C cell) origin. Moreover, the effect of GHS on the proliferation of three follicular-derived thyroid carcinoma cell lines (NPA, WRO and ARO) was also studied.

**Materials and Methods**

**Chemicals**

GHRP6 (His-d-Trp-Ala-Trp-d-Phe-Lys-NH$_2$), hexarelin (His-d-2Me-Trp-Ala-Trp-d-Phe-Lys-NH$_2$), Tyr-Ala-hexarelin and MK 0677 (N-[1(R){[1,2-dihydro-1-methanesulphonilspro-(3H-indole-3,4'-piperidin)-1-(yl)-2-(phenylmethoxy)-ethyl]-2-amino-2-methylpropanamide methane sulphonate) were provided by Europeptides (University of Naples). All the cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) – F12 medium supplemented with 10% foetal calf serum and penicillin/streptomycin in a 5% CO$_2$–humidified atmosphere at 37 °C and used to establish the effect of GHS on cell proliferation.

**Binding studies**

GHS binding sites were assayed on membranes (30 000 g pellet) isolated from human tissues or cell lines, as described previously (Muccioli et al. 1998) using $^{125}$I-labelled Tyr-Ala-hexarelin as ligand. This hexarelin analogue has been reported to have the same GH-releasing potency of hexarelin in rats (Ong et al. 1998b) and humans (Arvat et al. 1999), and to be a reliable probe for labelling in vivo GHS receptors in the human brain and pituitary gland (Muccioli et al. 1998, Ong et al. 1998b). For single point binding assay, tissue membranes (corresponding to 100 µg protein) were incubated in triplicate at 0 °C for 60 min with approximately $4 \times 10^{-9}$ mol/l $^{125}$I-labelled Tyr-Ala-hexarelin in a final volume of 0·5 ml assay buffer (50 mmol/l Tris, 2 mmol/l EGTA, 0·1% bovine serum albumin, 0·03% bacitracin, titrated with HCl to pH 7·3). Parallel incubations, in which $2·5 \times 10^{-6}$ mol/l unlabelled Tyr-Ala-hexarelin was also present, were used to determine non-specific 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 using a Packard auto-gamma counter. Specific binding was calculated as the difference between binding in the absence and in the presence of excess unlabelled Tyr-Ala-hexarelin and was expressed as a percentage of the total radioactivity added. To establish binding-site specificity, increasing concentrations of various competitors were tested in displacement assays with $^{125}$I-labelled Tyr-Ala-hexarelin.

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were harvested onto glass-fibre strips. Incorporation of \(^{3}H\)thyminidine was measured in a scintillation counter. For cell growth studies, thyroid carcinoma cell lines (8 \( \times \) \( 10^3 \) cells per ml) were seeded out in a 24-well plate containing DMEM–F12 medium supplemented with 10% foetal calf serum and grown for 96 h in the absence or in the presence of \( 1 \times 10^{-6} \) mol/l Tyr-Ala-hexarelin, GHRP6, MK 0677, GHRH or somatostatin. After incubation for 20 h – the time needed to obtain the maximal effect of the compounds – 1 µCi/well \(^{3}H\)thyminidine was added and the incubation was continued for a further 4 h. The reaction was then halted and the cells were harvested onto glass-fibre filter strips. Incorporation of \(^{3}H\)thyminidine was measured in a scintillation counter.

In selected experiments on cell lines, cells were synchronized 8 h after plating by a 36 h rest in 0.5% foetal calf serum. Cells were detached with trypsin-EDTA solution and counted double-blind by two independent investigators using a haemocytometer. All experiments were performed in triplicate.

**Statistical analysis**

Values are expressed as median and range unless otherwise noted. The number of cases is indicated by \( n \). Significant differences between groups were assessed by one-way ANOVA followed by Bonferroni or the Mann–Whitney test, depending on the experiments. \( P < 0.05 \) was chosen as the level of significance.

**Results**

**Binding of \(^{125}I\)-labelled Tyr-Ala-hexarelin to membranes from non-tumoral and neoplastic thyroid tissue**

The amount of \(^{125}I\)-labelled Tyr-Ala-hexarelin specifically bound to membranes of human thyroid gland and thyroid tumours of follicular and parafollicular (C cell) origin is reported in Table 1. Non-tumoral thyroid parenchyma showed appreciable Tyr-Ala-hexarelin binding values that were comparable to those previously found (11.3–14.0%) in a classical GHS target tissue, such as the human pituitary gland (Muccioli et al. 1998). Tyr-Ala-hexarelin specific binding was observed in all thyroid specimens examined and represented about 55–68% of total radioactivity bound. All follicular-derived tumours showed a specific binding also, with varying values according to the histological type. Tyr-Ala-hexarelin binding was remarkable in well differentiated (papillary and follicular) carcinomas and in poorly differentiated carcinomas, with values that were

<table>
<thead>
<tr>
<th>Histological type</th>
<th>Specific binding of (^{125}I)-labelled Tyr-Ala-hexarelin (% of radioactivity added/0.1 mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-tumoral thyroid</td>
<td></td>
</tr>
<tr>
<td>Thyroid parenchyma (( n = 5 ))</td>
<td>10.2 (8.3–12.2)</td>
</tr>
<tr>
<td>Thyroid capsule (( n = 3 ))</td>
<td>0.13 (0.05–0.2)</td>
</tr>
<tr>
<td>Follicular-derived tumours</td>
<td></td>
</tr>
<tr>
<td>Follicular adenomas* (( n = 9 ))</td>
<td>9.4 (7.7–13.0)</td>
</tr>
<tr>
<td>Papillary carcinomas (( n = 13 ))</td>
<td>34.0 (21.6–40.0)</td>
</tr>
<tr>
<td>Follicular carcinomas* (( n = 4 ))</td>
<td>26.7 (19.3–27.4)</td>
</tr>
<tr>
<td>Poorly differentiated carcinomas (( n = 2 ))</td>
<td>17.4 (16.3–18.5)</td>
</tr>
<tr>
<td>Anaplastic carcinomas (( n = 2 ))</td>
<td>4.1 (3.5–4.7)</td>
</tr>
<tr>
<td>Parafollicular (C cell)-derived tumours</td>
<td></td>
</tr>
<tr>
<td>Medullary carcinomas (( n = 6 ))</td>
<td>0.15 (0.1–0.4)</td>
</tr>
</tbody>
</table>

*Four of nine adenomas and two of four carcinomas were comprised of oxyphilic cells.
two to three times greater (P<0.05) than those in the thyroid gland, whereas anaplastic carcinomas showed specific binding values that were about 50% of those detected in non-tumoral thyroid tissue. In contrast, scant or negligible Tyr-Ala-hexarelin binding was observed in the thyroid capsule and in all parafollicular (C cell)-derived medullary carcinomas studied. A detectable specific binding of 125I-labelled Tyr-Ala-hexarelin was also present in three immortalized human cell lines that were originated from a papillary (NPA), follicular (WRO) and anaplastic carcinoma (ARO) of the thyroid. The binding values (mean ± s.e.m. of three separate determinations) were 14±1±1.0% for NPA, 12±1±1.8% for WRO and 7±3±1.2% for ARO cells.

Specificity of binding and saturation studies

To determine whether the binding of 125I-labelled 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 non-tumoral and neoplastic (papillary carcinomas) thyroid tissue that yielded sufficient amounts of membranes for these studies. The specificity of 125I-labelled Tyr-Ala-hexarelin binding to tissue membranes was established by determining the ability of different compounds to compete with the radioligand for the binding sites (Fig. 1). Both unlabelled peptidyl (Tyr-Ala-hexarelin, hexarelin and GHRP6) and non-peptidyl (MK 0677) GHS completely displaced radiolabelled Tyr-Ala-hexarelin from binding sites of non-tumoral thyroid, but MK 0677 was significantly less potent than peptidyl GHS (Fig. 1a). The IC50 values (mean ± s.e.m. of three separate experiments) were (1.9 ± 0.3) × 10−8 mol/l for Tyr-Ala-hexarelin, (2.1 ± 0.2) × 10−8 mol/l for hexarelin, (2.4 ± 0.3) × 10−8 mol/l for GHRP6 and only (1.5 ± 0.4) × 10−8 mol/l for MK 0677. In contrast, no competition was observed in the presence of peptides (GHRH and somatostatin) structurally unrelated to GHS. The pattern of displacement specificities in the neoplastic thyroid resembled that of the non-tumoral thyroid tissue (Fig. 1b). Experiments using increasing concentrations of radio-iodinated Tyr-Ala-hexarelin, ranging from 0.25 × 10−9 to 20 × 10−9 mol/l, revealed evidence of saturable specific binding in non-tumoral and neoplastic (papillary carcinoma) thyroid tissue (Fig. 2). Scatchard analysis of these data (Fig. 2, upper panel) demonstrated the existence, in both tissues, of a single class of high-affinity sites (Kd values 3.5 ± 10−9 mol/l for non-tumoral thyroid gland and 4.0 ± 10−9 mol/l for papillary carcinoma) with limited binding capacity (Bmax values 1232 fmol/mg protein for non-tumoral thyroid gland and 3185 fmol/mg protein for papillary carcinoma). The calculated Bmax values of Tyr-Ala-hexarelin binding sites in five papillary carcinomas were significantly greater (mean ± s.e.m. of 2483 ± 380 fmol/mg protein) than those measured in three non-tumoral thyroid glands (1232 ± 32 fmol/mg protein). However, the Kd values of the two tissues were not substantially different from one another, being (4.4 ± 0.6) × 10−9 mol/l in the papillary carcinomas and (3.8 ± 0.3) × 10−9 mol/l in the thyroid gland.

Effect of GHS on [3H]thymidine incorporation and cell proliferation

Based on the evidence of specific GHS binding sites in both non-tumoral and neoplastic thyroid, we investigated...
the effects of different GHSs, GHRH and somatostatin on the proliferation of three different human follicular thyroid carcinoma cell lines (NPA, WRO and ARO) in which the presence of specific GHS binding sites was also demonstrated. Figure 3 shows the effects of these compounds on the \( ^{3}H \)thymidine incorporation by NPA cells derived from a well differentiated thyroid papillary carcinoma; both the basal (serum-free conditions) and foetal calf serum-stimulated \( ^{3}H \)thymidine incorporation were studied. Both peptidyl (Tyr-Ala-hexarelin, GHRP6) and non-peptidyl (MK 0677) GHS were able to inhibit in a dose-dependent fashion the serum-stimulated \( ^{3}H \)thymidine incorporation, but MK 0677 was less effective than peptidyl GHS. The calculated 50% effective doses (mean ± S.E.M. of three separate experiments) were \((4.4 ± 0.3) \times 10^{-8}\) mol/l for Tyr-Ala-hexarelin, \((5.1 ± 0.6) \times 10^{-8}\) mol/l for GHRP6, and only \((3.3 ± 0.7) \times 10^{-7}\) mol/l for MK 0677. In contrast, no inhibition was observed in the presence of GHRH and somatostatin that did not compete with \( ^{125}I \)Tyr-Ala-hexarelin for the binding sites. Finally, there was also no change in thymidine incorporation when GHRH, somatostatin or different GHSs were incubated with NPA cells growing in serum-free conditions. Tyr-Ala-hexarelin, GHRP6 and MK 0677 also induced, in concentrations of \(1 \times 10^{-6}\) mol/l, a significant inhibition of cell growth in NPA cells: from the earliest time of treatment (24 h), the compounds were associated with a decrease in cell number (ranging from a 39 to a 43% inhibition) compared with controls. At 96 h, the inhibition was still significant, although it never exceeded a 30% decrease (Fig. 4a).
evident inhibition of cell growth induced by the above compounds was also observed in WRO cells that originated from a thyroid follicular carcinoma. In these cells, the growth inhibition (ranging from a 39 to a 45% decrease) was significant for all GHS at 24 and 48 h of treatment, but vanished at 96 h of incubation (Fig. 4b). Similarly, the inhibitory effect of GHS on the growth of the ARO (thyroid anaplastic carcinoma) cell line, observed at 24–48 h, was no longer recorded after 96 h of treatment when, in contrast, a significantly greater number of cells (27% increase) was found compared with controls (Fig. 4c). Cell growth experiments made at a different time with synchronous NPA, WRO or ARO cultures in the presence of different GHS yielded similar results, indicating the reproducibility of the above data. In all thyroid cancer cell lines studied GHRH and somatostatin had no effect on the cell proliferation (Fig. 4a, b and c).

Discussion

The existence of specific receptors for synthetic GH secretagogues (GHS-R) in peripheral tissues has been reported previously (Guan et al. 1997, Muccioli et al. 1997b, 1999, Ong et al. 1998a, Deghenghi et al. 1999). In previous studies on the distribution of peptidyl GHS binding in peripheral human tissues, we have found a specific binding in several endocrine and non-endocrine organs. Among endocrine tissues, significant peptidyl GHS binding was present in the thyroid gland (Muccioli et al. 1997b), with values comparable to those previously reported in the pituitary gland (Muccioli et al. 1998). Interestingly, the thyroid gland also expressed the gene encoding for GPR 38, an orphan receptor showing homology with the GHS-R type Ia expressed in the pituitary and central nervous system (McKee et al. 1997).

In agreement with these data, the present report confirms the existence of specific receptors for GHS in normal thyroid gland and demonstrates, for the first time, that these binding sites are present in thyroid cancers of follicular origin. The specific binding of 125I-labelled Tyr-Ala-hexarelin to membranes from well and poorly differentiated follicular tumours of the thyroid was significantly greater than that recorded in the non-tumoral thyroid parenchyma, low in anaplastic carcinomas and negligible in the parafollicular-derived medullary carcinomas. The reasons underlying these differences are unclear, although the present results suggest that the number of GHS-R in the thyroid is related to tumour differentiation and the embryological origin. Interestingly,

Figure 4 Effect of Tyr-Ala-hexarelin, GHRP6, MK 0677, GHRH and somatostatin on the proliferation of three human thyroid carcinoma cell lines (NPA, WRO and ARO) originated from a papillary carcinoma (NPA), a follicular carcinoma (WRO) and an anaplastic carcinoma (ARO) of the thyroid. NPA (a), ARO (b) and ARO (c) cells were grown for 96 h in the absence (○) or in the presence of 1 × 10^-6 mol/l Tyr-Ala-hexarelin (●), GHRP6 (□), MK 0677 (△), GHRH (▲) or somatostatin (▼) and counted every 24 h. Values are mean ± S.E.M. of three separate experiments. Significant differences from the corresponding value of control cells: *P<0.01, **P<0.001.
somatostatin binding sites were demonstrated in parafollicular-, but not in follicular-derived carcinomas (Reubi et al. 1991, Mato et al. 1998).

125I-Labelled Tyr-Ala-hexarelin binding in the non-tumoral and neoplastic thyroid tissues showed properties typical of the ligand–receptor interaction, such as high affinity, saturability and specificity. In particular, the binding of radioligand to membranes of these tissues was inhibited by unlabelled peptide (Tyr-Ala-hexarelin, hexarelin, GHRP6) and non-peptide (MK 0677) GHS, but not by neuropeptides (GHRH and somatostatin) structurally unrelated to GHS. This binding property overlaps with that reported in the pituitary and the hypothalamus (Muccioli et al. 1998, Ong et al. 1998b). However, it differs from that of other GHS target organs, such as the heart, in which the specific 125I-labelled Tyr-Ala-hexarelin binding is weakly inhibited by MK 0677 (Muccioli et al. 1997b, 1999, Ong et al. 1998a, Deghenghi et al. 1999). These data support the observation that more than one GHS-R subtype may exist in different peripheral organs (Howard et al. 1996, McKee et al. 1997, Ong et al. 1998b).

Specific GHS binding sites were also demonstrated in three human thyroid carcinoma cell lines (NPA, WRO and ARO). We therefore investigated the effects of GHS on the growth of these cell lines, showing that Tyr-Ala-hexarelin, GHRP6 and MK 0677 are able to inhibit serum-stimulated thymidine incorporation in a well differentiated thyroid papillary carcinoma cell line (NPA) at concentrations close to their binding affinity. These findings suggest that, in these experimental conditions, the above molecules possess an antiproliferative effect that could be mediated by the activation of specific receptors. The GHS also caused a significant inhibition in the growth of the NPA cell line, at a concentration of $1 \times 10^{-6} \text{ mol/l}$. This effect (ranging from a 39 to a 43% inhibition) was apparent from the earliest time point of incubation (24 h) and then decreased to 30% inhibition after 96 h of treatment. Similar results were obtained in a study of the effect of GHS on the growth of WRO cells derived from a thyroid follicular carcinoma, or the ARO cells originated from a thyroid anaplastic carcinoma. The antiproliferative effect of GHS on the WRO cell line was maximal (45% inhibition) at 24 h, but vanished at 96 h. Similarly, the inhibitory effect of GHS on the growth of ARO cells, observed at 24–48 h, was no longer recorded after 96 h of treatment when, in contrast, a significantly greater number of cells (27% increase) was found than with controls. This last finding suggests that GHS-R could also mediate a stimulatory effect on thyroid carcinoma cell proliferation, depending on both tumour cell differentiation grade and duration of exposure.

In conclusion, specific GHS binding sites were demonstrated in both non-tumoral and neoplastic human thyroid tissue. In addition, a growth inhibitory effect of GHS on follicular-derived thyroid cancer cell lines was found. In all, these findings confirm previous observations that GHS possess biological activities in organs other than the hypothalamus and pituitary (Berti et al. 1998, Desaphy et al. 1998, Isgaard et al. 1998, Bisi et al. 1999).

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