Growth hormone (GH) regulation of submandibular gland structure and function in the GH-deficient rat: upregulation of haptocorrin

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Abstract

Based on localization studies of the GH receptor/binding protein (BP) in the gastrointestinal tract, we have recently demonstrated growth hormone regulation of gastric intrinsic factor. In order to define the role of GH in the submandibular gland (SMG) we have investigated the effect of GH on SMG structure and function with particular reference to haptocorrin.

Bovine GH (65 µg/100 g body weight) was administered twice daily to adult male dwarf rats for 6 days (DW+) while control animals received vehicle (DW-). Administration of GH produced a significant increase in body weight (P < 0.001) and an allometric increase in SMG weight (P = 0). There was no change in RNA or protein content per g SMG and GH administration produced a small decrease in DNA content normalized to SMG weight. Morphometric analysis of the SMG revealed a significant increase in the percentage area of the gland occupied by tubular (GH receptor/BP expressing) structures and a significant increase in the diameter of both the intralobular striated and granular convoluted tubules. The effect of GH on cellular proliferation in the ductular and acinar components was determined by the immunohistochemical detection of nuclear 5'-bromo-2'-deoxyuridine (BrdU) incorporated during a 2-h pulse of BrdU. GH treatment induced a 5.5-fold increase in the labelling index of tubular cells whereas the acinar cell labelling index increased only 3.3-fold.

Soluble extracts of SMG were prepared for estimation of 57Co-cyanocobalamin (vitamin B12) binding. GH administration resulted in an increase in total 57Co-cyanocobalamin (CBL) binding per mg SMG protein. To determine the contribution of haptocorrin (R-protein) the amount of cobinamide dicyanide (CD) displaceable binding was calculated. GH administration produced a 70% increase in CD displaceable CBL binding per mg SMG indicating GH regulation of haptocorrin. A comparison of total SMG CBL binding and CD displaceable CBL binding between male and female rats detected no sex difference. Therefore sex-specific GH secretory profiles are unlikely to be of importance in the regulation of haptocorrin.

In conclusion we have demonstrated that GH stimulates hypertrophy and hyperplasia of components of the SMG in the dwarf rat. The observed upregulation of haptocorrin may synergize with the GH-stimulated increase in intrinsic factor to facilitate absorption of CBL during the anabolic state.

Introduction

Based on localization studies of the growth hormone (GH) receptor/binding protein (BP) (Lobie et al. 1990) in the gastrointestinal tract, we have recently demonstrated GH regulation of gastric intrinsic factor in the GH-deficient dwarf rat (Lobie et al. 1992). The increase in gastric intrinsic factor was also accompanied by hyperplasia of the gastric mucosa and subsequent increases in gastric epithelial height and mucosal surface area (Lobie et al. 1992). One other digestive tissue which expresses both the GH receptor (Lobie et al. 1990) and cobalamin binding proteins (Lee et al. 1989) in the rat is the submandibular gland. In the submandibular gland, the GH receptor is expressed solely on ductular (intralobular striated duct and granular convoluted tubule) and not acinar components (Lobie et al. 1990). The cells of the ductular structures are also those which produce the salivary gland cobalamin binding protein, R-protein or haptocorrin (Lee et al. 1989). This protein can be distinguished from the gastric intrinsic factor by its ability to bind a cobalamin analogue, cobinamide dicyanide (Allen 1975).

The submandibular gland (SMG) of the rat is sensitive to pituitary gland removal (Gresik 1994). In hypophysectomized animals, the SMG weight is markedly reduced (Baker & Abrams 1955, Koerker 1967, Liu & Lin 1969, see Gresik 1994 for review) and protein metabolism is curtailed (Sreenby 1953, Kronman & Chauncey 1964).
GH, among other hormones (Baker & Abrams 1955, Bixler et al. 1959, Liu & Lin 1969, Gresik 1994), has been reported to restore SMG weight towards normal in hypophysectomized animals. Atrophy of the tubular (Shafer et al. 1959, Bixler et al. 1959, Kronman & Chauncey 1964) and acinar (Sklar & Chauncey 1963) components upon hypophysectomy has also been reported. Morphologically the duct cells showed a reduction in cell height (Shafer et al. 1959) and in RNA and intracytoplasmic granular content (Sreenby 1953, Shafer et al. 1959). The effect of GH on the morphology and function of the SMG has not been described in detail although GH has been reported to restore towards normal the diameter of the tubular components and the intralobular striated duct in particular (Liu & Lin 1969).

We have, therefore, utilized the GH-deficient Lewis dwarf rat (Charlton et al. 1988, Skottner et al. 1989) to study the effect of GH on the structure and function of the SMG with particular reference to haptocorrin. We show that GH stimulates hyperplasia and hypertrophy of components of the SMG with a concomitant increase in haptocorrin (R-protein) content.

Materials and Methods

Materials

Recombinant bovine (b) GH was a generous gift from Monsanto Co. (St Louis, MO, USA). $^{57}$Co-cyanocobalamin, anti–5′-bromo-2′-deoxyuridine (BrdU) monoclonal antibody, sheep antimouse immunoglobulin G and the avidin–biotin horseradish peroxidase complex were purchased from Amersham (North Ryde, Australia). Cyanocobalamin, cobinamide dicyanide, pepsin from porcine gastric mucosa, 3′-3′-diaminobenzidine and BrdU were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Experimental design

Lewis dwarf (DW) male rats from a colony at the University of Queensland (Department of Physiology) were used in this study. DW rats have isolated GH deficiency (Charlton et al. 1988) and respond to exogenous GH treatment with marked increases in growth rate (Skottner et al. 1989). Animals were caged (two per cage) in a room at 22°C with a 12 h (0600 to 1800 h) light:darkness cycle with commercial rat pellets and DW rat supplement (a biscuit of complan, crushed oats and sunflower seeds) available ad libitum. Twelve twice daily i.p. injections of recombinant bGH (65 µg/100 g body weight) were given at 0700 and 1900 h to experimental animals (DW+). Control dwarf rats (DW−) were given vehicle (saline). Weight gain and food intake were monitored daily. Food was removed 4 h before termination. Animals were asphyxiated with CO$_2$ between 1100 and 1200 h (4 h after the 12th injection) to avoid potential variation in diurnal rhythm. Each experimental group consisted of a minimum of six animals.

Histomorphometry

After asphyxiation animals were perfused intracardially with PBS until blanching and then with cold Bouin’s solution (0·9% (v/v) picric acid, 9% (v/v) formaldehyde and 5% (v/v) acetic acid). The right submandibular gland was removed, its orientation marked and it was fixed by immersion in Bouin’s solution for 4 h at 4°C. Tissues were then embedded in paraffin by a standard histological procedure. All experimental groups were treated simultaneously with the same solutions/materials to avoid differential shrinkage. At least 10 sections were cut from the mid portion in the longitudinal plane of the anatomical position of the gland and stained with haematoxylin and eosin. Parameters were assessed using a digitizing tablet and the programme MEASURE (Capricorn Scientific Software, Woori Yallock, Victoria, Australia). Histological sections were projected via a mirror onto the tablet using a Leitz Prado microscope. All parameters were assessed blindly using a coded system which was revealed to the examiner after results were obtained. Sufficient measurements (usually 200 measurements per parameter per animal) were taken such that the parameter concerned possessed a standard deviation of not more than 10% of the final value for an individual animal (Lobie et al. 1992).

BrdU labelling of S-phase cells

Experimental animals were given an i.p. injection of BrdU (40 mg/kg body weight in PBS, pH 7·4) 2 h before death, perfused with Bouin’s fixative as described above and the submandibular glands were embedded in paraffin. Five micrometer sections were deparaffinized and washed in PBS and incorporation of BrdU assessed by the following schedule: (1) elimination of endogenous peroxidase activity with 0·1% H$_2$O$_2$ in methanol for 10 min; (2) digestion of sections with 500 U/ml porcine pepsin in 0·01 M HCl for 20 min at 37°C; (3) incubation with anti–BrdU monoclonal antibody in PBS–1% BSA (dilution 1:30) for 12 h at 4°C; (4) incubation with sheep anti-mouse biotinylated immunoglobulin G (diluted 1:100 in PBS–1% BSA) for 2 h at 25°C; (5) incubation with streptavidin–biotin horseradish peroxidase complex (diluted 1:100 in PBS–1% BSA) for 1 h at 25°C; (6) treatment with 3′-3′-diaminobenzidine (0·5 mg/ml) in PBS containing 0·1% H$_2$O$_2$. Between steps 2–6 sections were washed 3 times in PBS and once in PBS–1% BSA. Sections were counterstained in Mayer’s haematoxylin, dehydrated and mounted. Results were analysed by digitization as described above. Results are presented as the percentage of BrdU-labelled nuclei.
Termination and removal of SMG for biochemical measurements

After termination by CO₂ asphyxia, the right SMG was removed, frozen in liquid nitrogen and stored at −80 °C until sample processing.

DNA, RNA and protein estimations

SMG DNA, RNA and protein concentrations were determined as described by Dembinski and Johnson (1985) except that DNA concentration was determined by the method of Labarca and Paigen (1980) and protein by the method of Lowry et al. (1951). RNA was solubilized in 0·3 M KOH, DNA was solubilized in 10% perchloric acid and protein was solubilized in 1 m NaOH. The amounts of DNA, RNA and protein were normalized to SMG wet weight.

Preparation of SMG homogenates

SMGs were allowed to thaw in 6 vol. PBS, pH 7·4 before thorough homogenization with a polytron homogenizer. The homogenate was centrifuged at 15 000 g for 15 min and the supernatant removed and stored at −80 °C until assayed for R-protein.

57Co-cyanocobalamin binding

Cyanocobalamin (CBL) binding was determined according to Gottlieb et al. (1965) as modified by Adrian et al. (1989). The incubation volume was 1 ml consisting of 650 µl PBS, pH 7·4, 150 µl SMG homogenate, 100 µl PBS, pH 7·4 or 100 µl 10 µg/ml cobinamide dicyanide and 100 µl 57Co-CBL (600 fmol in PBS, pH 7·4). Incubation time was 40 min at 25 °C and the assay was terminated by addition of 1 ml haemoglobin (Hb)-coated activated charcoal (25 mg/ml bovine Hb with 250 mg/ml Norit A) with a further 10 min incubation at 25 °C before centrifugation for 5 min at 13 000 g. A 1 ml aliquot of the supernatant was taken and counted for 1 min in an LKB 1277 gamma-spectrometer. Assays were performed at a protein concentration such that the binding was in the linear range. Assays in which the 15 000 g supernatant was excluded or incubated with excess unlabelled cyanocobalamin did not contain detectable radioactivity. All values were normalized to protein concentration in the 15 000 g supernatant.

Statistical analysis

All data are expressed as means ± s.d. or s.e.m. Data were analysed using the two-tailed t-test or analysis of variance with Duncan’s multiple range test. Results were considered significant at the 5% level.

Results

Weight gain, food intake and SMG weight

Weight gains over the 6-day period of bGH administration are shown in Table 1. DW+ animals showed a significant (P<0·01) increase in weight over the treatment period whereas DW- animals did not (P=0·19). Food intake did not differ significantly between the two groups which obviated any requirement for pair feeding. As a consequence, food efficiency conversion (weight gain/food intake) was elevated in the DW+ group. The SMG weight was also significantly increased in the DW+ group compared with DW- but this increase was allometric as the SMG/body weight ratio did not differ between the two groups (Table 2).

DNA, RNA and protein levels

Changes in SMG DNA, RNA and protein levels between DW- and DW+ animals are shown in Table 2. There were small but not significant increases in total DNA, RNA and protein in the DW+ compared with the DW- group. When normalized to SMG weight there were no significant differences in the DNA, RNA or protein content of the SMG gland.

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DW-</th>
<th>DW+</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-experimental body weight</td>
<td>149 ±4</td>
<td>140 ±5</td>
<td>NS</td>
</tr>
<tr>
<td>Post-experimental body weight</td>
<td>155 ±3</td>
<td>165 ±6</td>
<td>&lt;0·01</td>
</tr>
<tr>
<td>Percentage weight increase</td>
<td>4·0 ±1·2</td>
<td>17·8 ±1·1</td>
<td>&lt;0·001</td>
</tr>
<tr>
<td>Food intake (g/rat/day)</td>
<td>13·3 ±0·2</td>
<td>13·6 ±0·3</td>
<td>NS</td>
</tr>
<tr>
<td>Food efficiency conversion</td>
<td>0·08 ±0·01</td>
<td>0·31 ±0·02</td>
<td>&lt;0·001</td>
</tr>
</tbody>
</table>

NS, not significant.
Morphometry of SMG

GH receptor expression in the rat SMG is restricted to the ductular components (intralobular striated ducts and granular convoluted tubules) (Lobie et al. 1990). Several related morphometric parameters including the percentage SMG are covered by ductular cell types (i.e. the tubulo-villous ratio) and the diameter of the intralobular (striated) ducts and granular convoluted tubules were therefore measured. bGH administration produced a 92 ± 9% increase in the area covered by the tubular cell types (both striated ducts and granular convoluted tubules) ($P$=0) (Fig. 1). To determine whether this effect was solely due to cellular hyperplasia (as evidenced by the BrdU labelling index) or also contained an element of cellular hypertrophy, the diameter of the ductular structures was measured. The diameters of both the striated ducts and granular convoluted tubules were significantly increased by GH treatment ($P$=0·025 and $P$=0·018 respectively) (Fig. 1). Thus GH acts predominantly on the GH receptor expressing ductular components of the rat SMG.

BrdU labelling of S-phase nuclei

BrdU is a non-radioactive thymidine analogue that is incorporated into DNA during the S-phase of the cell cycle. The effect of bGH on the incorporation of BrdU was investigated in both the acinar (non-GH receptor/BP expressing) and ductular (GH receptor/BP expressing) component of the SMG. BrdU labelling was evidenced by the deposition of brown peroxidase-diaminobenzidine reaction product after labelling with a BrdU-specific monoclonal antibody. The reaction was always nuclear and no deposition was observed in animals not injected with BrdU. bGH administration increased the labelling index of both the ductular and acinar components of the SMG (Fig. 2). However the magnitude of the GH response differed between the acinar and ductular components. GH induced a 5-5-fold increase ($P$=0) in the labelling index of ductular cells whereas the acinar cell (receptor negative) labelling index increased only 3-3-fold ($P$=0).

We have previously demonstrated GH regulation of gastric intrinsic factor (IF) in the DW rat (Lobie et al. 1992). The predominant CBL binding protein in the rat SMG is R-protein. However, the presence of IF has also been detected by immunohistochemistry and western blot analysis in the SMG (Dieckgraefe et al. 1988) and we have recently demonstrated SMG IF mRNA expression (J Garcia-Aragon, PE Lobie & MJ Waters, unpublished data). Therefore, we first determined the effect of bGH on total CBL binding in the SMG of the dwarf rat. bGH administration significantly increased total CBL binding per mg protein in the 15 000 g supernatant of SMG homogenates in DW+ compared with DW- animals (Fig. 3). To determine the contribution of R-protein to CBL binding in the SMG extracts, samples were also incubated with or without excess unlabelled (1 µg/ml) cobinamide dicyanide which binds R-protein but not IF (Allen 1975). We have previously demonstrated the validity of this approach to differentiate between the two proteins in gastric mucosa. bGH administration to DW+ animals resulted in a significant increase in cobinamide dicyanide displaceable binding ($P$=0·004) (Fig. 3) comparable to that observed with the effect on total CBL binding. The residual non-cobinamide displaceable CBL binding (presumably IF) was too low and variable to obtain any accurate data.

Effect of sex on CBL binding in SMG

To determine whether the male or female secretory pattern of GH affected the expression of R-protein in the SMG we compared the level of CBL binding in male and female SMGs. There was no significant difference in either total cobalamin binding ($P$=0·65) or in cobinamide dicyanide displaceable CBL binding ($P$=0·87) between male and female rats (Fig. 4) suggesting no involvement of GH secretory pattern in the GH regulation of R-protein.

**Table 2** Effect of 6 days treatment of dwarf rats without (DW-) or with (DW+) recombinant bGH on submandibular gland weight and DNA, RNA and protein content

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DW-</th>
<th>DW+</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mg)</td>
<td>136 ± 4</td>
<td>152 ± 2</td>
<td>$&lt;0·001$</td>
</tr>
<tr>
<td>Total DNA (µg)</td>
<td>128 ± 11</td>
<td>139 ± 16</td>
<td>NS</td>
</tr>
<tr>
<td>DNA (µg/g SMG)</td>
<td>943 ± 29</td>
<td>916 ± 26</td>
<td>NS</td>
</tr>
<tr>
<td>Total RNA (mg)</td>
<td>2·02 ± 0·03</td>
<td>2·17 ± 0·3</td>
<td>NS</td>
</tr>
<tr>
<td>RNA (µg/g SMG)</td>
<td>14·68 ± 0·23</td>
<td>13·66 ± 1·14</td>
<td>NS</td>
</tr>
<tr>
<td>Total protein (mg)</td>
<td>10·2 ± 0·6</td>
<td>11·7 ± 2·2</td>
<td>NS</td>
</tr>
<tr>
<td>Protein (mg/g SMG)</td>
<td>75·1 ± 4·8</td>
<td>71·5 ± 4·5</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, not significant.
Discussion

We have investigated the effect of GH administration on SMG structure and function in the dwarf rat as a result of our localization of the GH receptor/BP in this gland (Lobie et al. 1990) and the demonstration of GH regulation of gastric IF (Lobie et al. 1992). The SMG, like the gastric mucosa, provides a good model to study in vivo effects of...
GH since they both contain discrete GH receptor expressing and non-expressing cell types. We have used a defined in vivo system for our study on the regulation of SMG structure and function. Dwarf rats are specifically GH-deficient with other pituitary hormones present at normal levels (Charlton et al. 1988). This is important since other hormones such as thyroid hormone and androgens exert major effects on the SMG (Gresik 1994, Kurihara et al. 1996). Recombinant bGH was again used so that the response obtained was somatogenic and not lactogenic or a result of contamination by other pituitary hormones. It is interesting that GH stimulates the proliferation of both the acinar and ductular components of the SMG. The GH receptor is expressed on the ductular component of the SMG (Lobie et al. 1990) and, presumably, at least some of the mitogenic response is mediated by a direct action of GH. The direct action may involve local production of insulin-like growth factor-I (IGF-I) since IGF-I is expressed in the ductular cells (Hansson et al. 1988) and we have shown that IGF-I mRNA levels in the SMG are regulated by GH (J Garcia-Aragon, PE Lobie, MJ Waters, unpublished observations). The acinar component of the gland contains no detectable GH receptor and thus the mitogenic response must come from either locally produced IGF-I or endocrine IGF-I or some other GH-induced protein which acts in a paracrine or endocrine manner. In any case the predominant mitogenic response in the SMG resides in the ductular cells though it should be remembered that the BrdU labelling represents only a 2-h pulse over 6 days of treatment with bGH. This is further exemplified by the fact that GH administration increased the proportion of tubular to acinar structures in the SMG. Such an increase in the proportion of tubular to acinar structures in the SMG is also observed during the postnatal development of the gland (Jacoby & Leeson 1958), an effect now potentially mediated by GH. The function of the increase in tubular structures is not obvious but may relate to a maturation of the gland to produce growth stimulatory substances such as epidermal growth factor (EGF), nerve growth factor and IGF-I. Sialadenectomy has been reported to result in growth impairment of the animal but it is difficult to dissociate the contribution of the ‘endocrine’ and exocrine components of the SMG to growth of the animal.

We show here that GH increases the content of haptocorrin (R-protein) in the SMG of the dwarf rat, extending our earlier result demonstrating GH regulation of IF (Lobie et al. 1992). GH has also been demonstrated to maintain IF receptor number in the hysterectomized
Patients with congenital deficiencies of haptocorrin display a low serum vitamin B12 level but are otherwise asymptomatic (Carmel 1982, Zittoun et al. 1988) in comparison to IF-deficient patients.

We also investigated whether gender influenced the level of haptocorrin expression since GH exhibits sex-specific secretory profiles and these regulate sex-specific differences in many hepatic enzymes (Eden et al. 1987). No difference in the level of total cobalamin binding or in the level of cobinamide dicyanide displaceable cobalamin binding was observed between male and female rats. Thus, sex-specific secretory profiles of GH secretion are not involved in the regulation of haptocorrin synthesis. This is interesting since the SMG displays marked sexual dimorphism at least in terms of EGF content (Hirata & Orth 1979). The level of STAT5 expression is thought to be responsible for the sex-specific expression of some cytochrome P450 genes (Waxman et al. 1995). In this regard it will be interesting to determine the GH-activated transcription factors regulating expression of both the IF and haptocorrin genes. At least IF is regulated at the transcriptional level by GH (J Garcia-Aragon, P E Lobie, M J Waters, unpublished observations).

In conclusion, we have demonstrated hyperplasia and hypertrophy of the SMG of the dwarf rat in response to GH with a concomitant increase in the expression of haptocorrin. We propose that this increase is part of a general upregulation of the B12 axis induced by GH as part of the anabolic response.

References


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