Significance of carbohydrate residues in human chorionic gonadotropin (hCG) for expression of biological activity

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Abstract - Testosterone is produced in male rats by the action of luteinizing hormone (LH), an anterior pituitary hormone which interacts with receptors on Leydig cells (testes) to activate adenylate cyclase. A most frequently used agonist for LH is human chorionic gonadotropin (hCG), a hormone synthesized by the placenta during the early stages of pregnancy. Like LH, it is a heterodimer consisting of two non-covalently bound subunits designated as alpha and beta. Each subunit of LH/hCG contains oligosaccharide chains of varying sizes and intersugar linkages.

In order to determine the significance of carbohydrate residues of hCG in receptor interaction and signal transduction, deglycosylated hCG (DG-hCG), free of more than 80% of its sugar residues, was prepared by treatment of hCG with anhydrous HF. The effect of this derivative was tested on two different hCG responsive purified testicular interstitial cell fractions as described (Bhalla, V., Flasch, M., Browne, E., Sohal, G., and Sharawy, M. (1987) J. Biol. Chem. 262:5322). Fraction I non-steroidogenic light cells, previously found to bind 125I-labeled hCG with high affinity (Kd 3.0 x 10^-10M), also bound 125I-labeled DG-hCG with a similar high affinity (Kd 6.3 x 10^-10M) without stimulating testosterone production. The binding of 125I-labeled hCG to light cells was also inhibited by DG-hCG. Fraction IV steroidogenic Leydig cells, which produce cAMP and testosterone in response to hCG (2 ng/2 million cells/250 ul) without detectable high affinity binding sites for that hormone, neither bound DG-hCG with high affinity nor sufficiently produced cAMP and testosterone in the presence of DG-hCG alone. With addition of intact hCG (2 ng/2 million cells/250 ul), DG-hCG inhibited hCG stimulated cAMP levels by 70-90%, although this was insufficient to inhibit testosterone production even at higher DG-hCG concentrations. This observation was contrary to previous studies in which DG-hCG was shown to be an antagonist to hCG action in vitro. From this study we conclude that DG-hCG (a) retains its receptor binding activity in the non-steroidogenic light cells and this high affinity binding is unrelated to steroidogenesis, (b) like hCG, shows an extremely small number of high affinity binding sites on Leydig cells, (c) loses its biological activity as a result of deglycosylation, (d) actions in this study support the concept of two different hCG responsive cells in the rat interstitium which, if not separated, will yield misleading data supporting the coexistence of hCG high affinity binding and biological response in the same cell and (e) partially antagonizes the activation of adenylate cyclase but not enough to block testosterone production thus questioning the usefulness of DG-hCG in developing means to control and/or regulate fertility.

INTRODUCTION

Human chorionic gonadotropin (hCG), synthesized and secreted by human placenta, is a glycoprotein hormone composed of two non-covalently linked subunits designated as alpha and beta. Although the specificity resides in the beta subunit, when separated, the two individual subunits are biologically inactive. Both subunits contain N- and O-linked oligosaccharide chains which are attached to amino acids (asparagine and serine/threonine, respectively) of the polypeptide. The alpha subunit contains two N-linked oligosaccharide chains at positions 52 and 78 whereas on the beta subunit they are located on positions 13 and 30. In addition, there are four O-linked oligosaccharides attached to serine residues at positions 121, 127, 132, and 138 on the beta subunit (for review, see ref. 1). Recent studies have shown that the removal of the sugar residues from the hormone, either through chemical (2) or enzymatic (3) deglycosylation, can be selectively achieved without adversely affecting the protein backbone of the molecule.
The biological properties of hCG are similar to those of luteinizing hormone (LH) which is produced by the anterior pituitary gland and acts on Leydig cells in testis to stimulate the synthesis of testosterone, a male hormone essential for maintenance of spermatogenesis (for review see ref. 4). Neither of these hormones enter the cell but instead they bind to testicular receptors with high affinity ($K_d\sim 10^{-10}M$), activate adenylate cyclase to produce intracellular cAMP which, in turn, acts as a second messenger of hormone action to promote steroidogenesis (for review see ref. 5). Because LH is not readily available in purified form and is more susceptible to degradation during radioiodination, most of the structure function studies have been performed using hCG to explore the mechanism of hormone action.

It has recently been reported that deglycosylated hCG (DG-hCG), after removal of 70-80% of its sugar residues, binds to the hCG receptors with high affinity but otherwise loses its biological activity. Because the binding of the hormone is believed to be a prerequisite for induction of the biological response, efforts have been directed to exploit this derivative to control/regulate hormone action. DG-hCG also acts on the target cell in vitro as an antagonist by inhibiting intact hCG-induced cAMP production (6) as well as hCG-induced steroid production (7, 8). DG-hCG inhibits the action of the intact hormone stimulated cAMP levels by 80-90% whereas steroidogenesis is decreased by 50-75%. The mechanism of this inhibition of biological function at the level of the receptor is believed to be due to the occupancy of hCG binding sites by DG-hCG such that further occupancy of those sites by hCG is prevented and the biological action is obliterated due to DG-hCG occupancy of hCG binding sites, possibly causing a defect in coupling in signal transduction mechanism at the membrane level.

The foregoing studies were performed with intact testis and/or unpurified Leydig cells. We, however, recently succeeded in purifying two hCG-responsive cells from rat testis. The light cells bound $^{125}$I-labeled hCG with high affinity without generation of cAMP and testosterone whereas the heavier Leydig cells produced cAMP and testosterone in response to hCG stimulation without detectable high affinity binding sites (9-11). These two cell types were also morphologically distinct by electron microscopy and the $^{125}$I-labeled hCG binding sites localized on the light cells and absent from the Leydig cells were confirmed by autoradiography. The observations made with this system have drawn our attention to the possibility that not all of the high affinity binding sites for hCG on the target cell are physiologically coupled to signal transduction and that we have an ideal model to investigate the chemical properties of DG-hCG in purified cells in order to understand the mechanism of LH/hCG action in testis in vitro as described herein.

MATERIALS AND METHODS

Interstitial cells from rat testes were prepared by collagenase digestion as described previously (10). After collagenase digestion and filtration, the cell number was counted using a Coulter Counter (Model ZM, Coulter Electronics Ltd., Luton Beds, England). The viability of cells was routinely verified by trypan blue and erythrocyte B dye exclusion tests (12). Highly purified hCG (CR 122, 13,450 IU/mg) were obtained from the NIAMDD, Bethesda, MD. The highly purified and chemically characterized preparations of DG-hCG (2, 6) were obtained from Drs. M.R. Sairam and H. Chen.

The collagenase dispersed interstitial cells were purified by discontinuous Percoll gradient consisting of 5 ml of 25% Percoll (v/v), and 20 ml of 20% Percoll (v/v) in medium 199 supplemented with 4.2 mM sodium bicarbonate, 4.2 mM HEPES, 0.125 mM 3-isobutyl-xanthine (MIX), 0.1% bovine serum albumin, 1% streptomycin-penicillin, 0.1% gentamicin, and 1% fetal calf serum (pH 7.4) under the standard conditions recently reported (10). The light cell fraction (1-10 ml, fraction I) and the Leydig cell fraction (27-30 ml, fraction IV) were washed, counted, and resuspended in Medium 199 (20 x 10^6 cells/ml).

Aliquots of the purified light and Leydig cells (500 ul; 10 x 10^6 cells), were incubated with or without variable doses of hCG or DG-hCG as indicated in a total volume of 1.25 ml in M199 for 2 h at 34°C in a shaking water bath (170 oscillations/min under O_2:CO_2, 95:5%). These experimental conditions for hormone stimulation were similar to the conditions of the binding assay in which 2 x 10^6 cells were incubated in 250 ul in the presence and absence of hormone. Testosterone and cAMP produced in response to hCG, DG-hCG or a combination of the two in both compartments (released and intracellular) were measured by their respective radioimmunoassays as described previously (9, 10). Total product formed was obtained by adding these two values.

For the binding assay (9, 10), purified cells (2 x 10^6) were incubated with 2.5 ng of $^{125}$I-labeled hCG (50,000 cpm; 20 uCi/ug; prepared by chloramine T method, ref. 9) in the presence and absence of increasing concentrations of unlabeled hCG or DG-hCG (1.0-100 ng) in a total volume of 250 ul. The determinations were made in duplicate and the incubations were carried out at 37°C for 1 hr. Non-specific binding was determined by measuring cpm bound in the presence of excess unlabeled hCG (2000 ng/tube).
Statistical analyses were done using Duncan's multiple range test. A p value of 0.05 or less was considered significant. Where indicated, results are also expressed as Mean ± S.E.

RESULTS AND DISCUSSION

The specific binding of $^{125}$I-labeled hCG to light cells was 0.308 ng/2 x $10^6$ cells/250 ul and was inhibited by unlabeled hCG in a dose dependent manner (Fig. 1). The ED$_{50}$ was calculated to be $6.0 \times 10^{-10}$M hCG. This value was in close agreement with the affinity of $^{125}$I-labeled hCG binding ($K_d$, equilibrium dissociation constant, $3.0 \times 10^{-10}$M by Scatchard analysis). The specific $^{125}$I-labeled hCG binding to the Leydig cells was approximately 9 fold lower (0.036 ng/2 x $10^6$ cells/250 ul). There was erratic displacement of the labeled hormone by the unlabeled hCG, most likely caused by the low number of binding sites present on these cells. Assuming that the specific binding represents saturation high affinity receptor sites, the exceedingly small number of hCG receptors identified on active Leydig cells was 1.1 fmol/2 x $10^6$ cells/250 ul.

When deglycosylated hCG was tested for its ability to compete for $^{125}$I-labeled hCG binding sites on the light cells, it also displaced the labeled hCG binding in a dose dependent manner with a similar ED$_{50}$ ($8.4 \times 10^{-10}$M; Fig. 2). The $K_d$ value for this interaction was $6.3 \times 10^{-10}$M. With the Leydig cells, the DG-hCG displayed the same erratic pattern of displacement seen earlier with the unlabeled hCG. It was thus apparent that the DG-hCG interacted with the hCG binding sites on the light cells in a manner similar to that seen with intact hCG. These studies also supported the conclusion that the carbohydrate side chains are not required for the expression of the binding activity via hormone receptor interaction.

Figure 1. Competitive inhibition plot of specific $^{125}$I-labeled hCG binding to light and Leydig cells in the presence of unlabeled intact hCG. The light and Leydig cells (2x10$^6$ cells), purified by a discontinuous gradient of Percoll (10) were incubated with $^{125}$I-labeled hCG (2.5 ng/250 ul) in the absence and presence of increasing concentrations of unlabeled hCG for 1 hr. at 37°C and the tubes were processed as described under "Materials and Methods". Non-specific binding in the presence of excess unlabeled hormone was subtracted to obtain specific binding.

Figure 2. Competitive inhibition plot of $^{125}$I-labeled hCG binding to light and Leydig cells in the presence of increasing concentrations of unlabeled DG-hCG. The binding assays were performed as described under the legend for Fig. 1 except that unlabeled DG-hCG was used for competition for $^{125}$I-labeled hCG binding.

When the hormone responsiveness of the light and Leydig cells was tested, we found that the
light cells did not produce cAMP or testosterone in response to maximal hCG stimulation (2 ng/2 x 10^6 cells/ 250 ul; left panel, Figs. 3 and 4, respectively), as observed in previous studies (9-11). Therefore, no further experiments with hCG or DG-hCG with the light cells are presented in this study. On the other hand, Leydig cells responded to hCG and therefore the effect of DG-hCG was tested in greater detail.

The production of total cAMP and testosterone (cAMP/testosterone released into the medium and intracellular cAMP/testosterone) by incubation of Leydig cells with increasing concentrations of unlabeled hCG in vitro is shown in Figs. 3 and 4, respectively (right panel). Most of the cAMP formed in response to the hormone action, seen in Fig. 3, was excreted into the medium as shown in Table 1. The hCG-stimulated intracellular cAMP concentrations were in the range of 2 to 6 pmol/2 x 10^6 cells. Due to the very low K_m of cAMP for steroidogenesis (vide infra), these low concentrations of intracellular cAMP were sufficient to promote maximal de novo synthesis of testosterone (Fig. 4). The release of testosterone into the medium was consistent with the secretory nature of these steroidogenic cells (Table 1).

DG-hCG, by itself, caused a slight elevation in testosterone production (Fig. 4) which is attributable to residual hCG activity, although it did not stimulate cAMP production (Fig. 3). It is quite possible that the stimulated values for cAMP were so small that the assay failed to detect it.

Figure 3. cAMP production by light and Leydig cells in the presence of increasing concentrations of intact hCG or DG-hCG. Two million cells were incubated with indicated amount of unlabeled hCG or DG-hCG in 250 ul for 2 hours at 34°C and the tubes were processed to separate samples containing cAMP released in the medium and remaining in the cells. The levels of cAMP were measured by cAMP radioimmunoassays. In this figure, the cAMP in the two compartments is added to reflect total formation of the product after subtraction of basal levels. Levels of cAMP released into the medium are shown in Table 1.

Figure 4. Testosterone production by light and Leydig cells in the presence of increasing concentrations of intact hCG or DG-hCG. The experimental conditions were identical to those described under Fig. 3 and testosterone levels in the two compartments were measured by testosterone radioimmunoassays.

To test the antagonistic properties of DG-hCG, Leydig cells were incubated with a maximal concentration of intact hCG, in the absence or presence of increasing doses of DG-hCG. DG-hCG caused inhibition of hCG-stimulated cAMP production in a dose-dependent manner (Fig. 5) with 90% inhibition seen at a DG-hCG concentration of 200 ng/ 250 ul. In our hands, DG-hCG invariably inhibited hCG stimulated cAMP production in the range of 70-90%. We have recently found that the half-maximal intracellular cAMP concentration necessary for maximal steroidogenesis is 9.8 x 10^-10M (Browne and Bhalla, manuscript under preparation), thus
very small concentrations of intracellular cAMP (despite the inhibition of excess production of cAMP by DG-hCG) were sufficient to promote steroidogenesis (Table 2). The production of testosterone was unaffected even when DG-hCG in excess of 200 ng were used. Very similar conclusions were drawn when another preparation of DG-hCG (Sairam's), prepared by HF treatment, was utilized (13).

**TABLE 1.** Dose dependent release of hCG-stimulated cAMP and testosterone in the extracellular media.

<table>
<thead>
<tr>
<th>hCG Added (ng/2 x 10^6 cells)</th>
<th>Released Product (pmol/2 x 10^6 cells/250 ul)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>cAMP</td>
</tr>
<tr>
<td>0.2 ng</td>
<td>2.35 ± 2.04</td>
</tr>
<tr>
<td>0.4 ng</td>
<td>7.17 ± 1.49</td>
</tr>
<tr>
<td>2.0 ng</td>
<td>12.82 ± 1.53</td>
</tr>
<tr>
<td>4.0 ng</td>
<td>15.31 ± 1.54</td>
</tr>
<tr>
<td>6.0 ng</td>
<td>18.53 ± 1.94</td>
</tr>
<tr>
<td>10.0 ng</td>
<td>15.55 ± 1.30</td>
</tr>
<tr>
<td>100.0 ng</td>
<td>20.88 ± 1.62</td>
</tr>
<tr>
<td>200.0 ng</td>
<td>18.46 ± 1.90</td>
</tr>
</tbody>
</table>

**Fig. 5** Antagonistic properties of DG-hCG in decreasing hCG stimulated cAMP production in Leydig cells. The Leydig cells were stimulated with maximal concentration of hCG (2 ng/250 ul) in the absence and presence of increasing concentrations of DG-hCG under the conditions described previously (see Fig. 3 legend). Values for total cAMP inhibition are given.

**TABLE 2.** Effect of DG-hCG on hCG stimulated Testosterone Production by Leydig cells.a

<table>
<thead>
<tr>
<th>Hormone Added (ng/2 x 10^6 cells)</th>
<th>hCG Stimulated Testosterone Produced (pmol/2 x 10^6 cells/250 ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intracellular</td>
</tr>
<tr>
<td>2 ng hCG</td>
<td>1.87 ± 0.13</td>
</tr>
<tr>
<td>2 ng hCG + 2 ng DG-hCG</td>
<td>0.28 ± 0.14</td>
</tr>
<tr>
<td>2 ng hCG + 20 ng DG-hCG</td>
<td>0.56 ± 0.49</td>
</tr>
<tr>
<td>2 ng hCG + 100 ng DG-hCG</td>
<td>0.83 ± 0.31</td>
</tr>
<tr>
<td>2 ng hCG + 200 ng DG-hCG</td>
<td>0.59 ± 0.56</td>
</tr>
</tbody>
</table>

(a) The experimental condition were identical to those described under the legend for Fig. 5.
From this study we conclude that DG-hCG (a) retains its receptor binding activity in the non-steroidogenic light cells and this high affinity binding is unrelated to steroidogenesis, (b) binding sites or hCG binding sites in Leydig cells are present in very small number and the true affinity of the hormone-receptor sites can not be estimated by the current method, (c) loses its biological activity after deglycosylation, suggesting that carbohydrate residues are essential for the expression of hormone activity eventhough these residues are apparently not required for the hormone binding activity, (d) actions in this study support the concept of two different hCG responsive cells in the rat interstitium which, if not separated, will yield misleading data supporting the coexistence of hCG high affinity binding and biological response in the same cell, (e) partially antagonizes the activation of adenylate cyclase but the cAMP formation is not completely inhibited so that the minute quantities of cAMP are sufficient to promote de novo testosterone biosynthesis in purified Leydig cells and f) does not antagonize hCG-promoted steroidogenesis, thus questioning the usefulness of this derivative in regulation of fertility control. Our results are supported by two recent studies in which the authors doubt if DG-hCG can be used as an effective antagonist of endogeneous LH action in primate and also in human (clinical trial) studies (14, 15).

Acknowledgements

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REFERENCES