Molecular and Cellular Endocrinology 479 (2019) 103–109

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Hyperglycosylated hCG activates LH/hCG-receptor with lower activity than hCG

Hannu Koistinen[a,*], Mariann Koel[b,c], Maire Peters[b,d], Ago Rinken[e], Karolina Lundin[f], Timo Tuuri[f], Juha S. Tapanainen[f,g], Henrik Alfthan[a], Andres Salumets[b,d,f,h], Ulf-Håkan Stenman[a,1], Darja Lavogina[b,e,1]

[a] Department of Clinical Chemistry, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[b] Competence Centre on Health Technologies, Tartu, Estonia
[c] Department of Cell Biology, Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia
[d] Department of Obstetrics and Gynaecology, Institute of Clinical Medicine, University of Tartu, Tartu, Estonia
[e] Institute of Chemistry, University of Tartu, Tartu, Estonia
[f] Department of Obstetrics and Gynecology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[g] Department of Obstetrics and Gynecology, University Hospital of Oulu, University of Oulu, Medical Research Center Oulu and PEDEGO Research Unit, Oulu, Finland
[h] Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia

ARTICLE INFO

Keywords:
hCG
Hyperglycosylated hCG
LHCGR
LH/hCG receptor
Reporter system

ABSTRACT

While human chorionic gonadotropin (hCG) appears to have an essential role in early pregnancy, it is controversial whether the hyperglycosylated form of hCG (hCG-h), which is the major hCG isoform during the first 4–5 weeks of pregnancy, is able to activate LH/hCG receptor (LHCGR). To address this, we utilized different extensively characterized hCG and hCGβ reference reagents, cell culture- and urine-derived hCG-h preparations, and an *in vitro* reporter system for LHCGR activation. The WHO hCG reference reagent (99/688) was found to activate LHCGR with an EC_{50}-value of 3.3 ± 0.6 pmol/L (n = 9). All three studied hCG-h preparations were also able to activate LHCGR, but with a lower potency (EC_{50}-values between 7.1 ± 0.5 and 14 ± 3 pmol/L, n = 5–11, for all $P < 0.05$ as compared to the hCG reference). The activities of commercial urinary hCG (Pregnyl) and recombinant hCG (Ovitrelle) preparations were intermediate between those of the hCG reference and the hCG-h. These results strongly suggest that the hCG-h is functionally similar to hCG, although it has lower potency for LHCGR activation. Whether this explains the reduced proportion of hCG-h to hCG reported in patients developing early onset pre-eclampsia or those having early pregnancy loss remains to be determined.

1. Introduction

The glycoprotein hormones luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid stimulating hormone (TSH) and human chorionic gonadotropin (hCG)[1] are heterodimers consisting of a non-covalently linked common α-subunit (hCGα) and different β-subunits determining their biological activity (Pierce and Parsons, 1981). While TSH and FSH have specific receptors, hCG and LH signal through a common LH/hCG receptor (LHCGR), which is highly expressed in ovaries and testes, but also at low levels in some other tissues, like breast and uterus (reviewed in Ascoli et al., 2002). Ligand binding to a $G_{\alpha s}$ protein-coupled LHCGR activates the cAMP system and subsequently cAMP-dependent protein kinases, but some other signaling routes have also been reported (reviewed in Ascoli et al., 2002). The major function of hCG is to maintain progesterone production in the corpus luteum, essential for pregnancy.

In addition to the intact hCG heterodimer, the free β-subunit of hCG (hCGβ), which is produced especially in non-trophoblastic tumors (Stenman et al., 2004), has been proposed to be bioactive (Cole and Butler, 2012), although it does not activate the LHCGR (Catt et al., 1973; Ryan et al., 1988). We have recently shown that both hCG and hCGβ stimulate trophoblast invasion independent of the LHCGR (Lee et al., 2013). Especially in urine, which is often used as a source for purification of hCG and hCGβ, part of the hCGβ occurs as a

Abbreviations: cAMP, cyclic adenosine monophosphate; hCG, human chorionic gonadotropin; hCGβ, free β-subunit of hCG; hCGβn, nicked hCGβ; hCGβcf, hCGβ core fragment; hCG-h, hyperglycosylated hCG; LHCGR, LH/hCG receptor; LH, luteinizing hormone

* Corresponding author. Department of Clinical Chemistry, Biomedicum Helsinki, PO Box 63, 00014, University of Helsinki, Finland.
E-mail address: hannu.k.koistinen@helsinki.fi (H. Koistinen).

[1] Equal contribution.

https://doi.org/10.1016/j.mce.2018.09.006
Received 3 July 2018; Received in revised form 13 August 2018; Accepted 22 September 2018
Available online 02 October 2018

proteolytically processed, or nicked, isoform (hCGβn) in which the peptide chain is cleaved between Gly47 and Val48, and less often between amino acids 43–44, 44–45 and 75–76 (Bidart et al., 1988; Puisieux et al., 1990; Elliott et al., 1997). hCGβn fragments are connected by disulfide bridges and are able to form a heterodimer (hCGn) with α-subunit, but this dimer has a very low or no activity at the LHCGR (Sakakibara et al., 1990). hCGβ can be further processed in the kidneys to a hCGβ core fragment (hCGβcf), which, in addition to lacking several amino acids in the region 41–54, is also devoid of the five N-terminal amino acids as well as amino acids 93–145 forming the C-terminal peptide (CTP). hCGβcf is unable to form a dimer with hCGα (Birken et al., 1988; Nisula et al., 1989). Plasma contains very low levels of hCGn and hCGβcf (Hoermann et al., 1994; Alfthan et al., 1992).

Invasive cytotrophoblasts and several cancers, especially trophoblastic and testicular cancer, produce hyperglycosylated hCG (hCG-h) (Ryan et al., 1988; Valmu et al., 2006; Kovalevskaya et al., 1999, 2002a; Stenman et al., 2011; Lempiäinen et al., 2012). hCG-h is characterized by large glycan moieties and defined as hCG detected by a monoclonal antibody B152 that recognizes a core-2 glycan attached to Ser-132 of hCGβ and surrounding peptide structures (Valmu et al., 2006; Birken et al., 1999, 2003a). hCG-h is the major form of hCG found in serum during the first 4–5 weeks of pregnancy (Kovalevskaya et al., 1999; Stenman et al., 2011). Specific determination of hCG-h is clinically useful for cancer diagnostics and prognosis, but also for prediction of pregnancy complications (reviewed in Stenman et al., 2004; Stenman et al., 2006; Stenman and Alfthan, 2013), e.g., during the first trimester of pregnancy, a reduced proportion of hCG-h to hCG predicts early onset pre-eclampsia (Keikkala et al., 2013). In cancer diagnostics, hCG-h may be especially useful for detection of non-seminomatous germ cell tumors of the testis, where elevated concentrations also indicate adverse prognosis (Lempiäinen et al., 2012).

It has been suggested that hCG-h is functionally different from non-hyperglycosylated hCG (Cole, 2010; Fournier et al., 2015). Such suggested activities include stimulation of trophoblast and cancer cell invasion, and activation of the TGFβ-receptor (Cole and Butler, 2012; Handschuh et al., 2007; Evans et al., 2015; Berndt et al., 2013). However, in our studies on highly purified and well characterized hCG and hCGβ preparations, with different degrees of hyperglycosylation, we have not seen any clear glycosylation-related modulation of pro-invasive activity of hCG/hCG-h (Lee et al., 2013). Furthermore, activation of the TGFβ-receptor may be due to TGFβ-contamination in the hCG-h preparations used (Koistinen et al., 2015). Thus, it remains controversial whether hyperglycosylation of hCG has specific (patho)physiological significance or is just a consequence of the altered glycosylation in cancer and trophoblast cells of early pregnancy. While the activation of LHCGR by hCG is well established, studies on the activity of hCG-h on LHCGR activation have given discordant results (Berndt et al., 2013; Crochet et al., 2012; Mazina et al., 2015a), ranging from inactivity to somewhat reduced activity. This may be due to differences between various hCG preparations used and their poor characterization, but also the assays used. To address these contradictory findings, we studied LHCGR activation by several extensively characterized hCG preparations and report that hCG-h is able to activate the LHCGR, although with lower potency than hCG.

2. Materials and methods

2.1. hCG, hCGβ and their hyperglycosylated forms

WHO international reference preparations for hCG (code 99/688), nicked hCG (99/642), hCGβ (99/650) and nicked hCGβ (99/692) were obtained from National Institute for Biological Standards and Control (NIBSC, Potters Bar, Hertfordshire, UK). Recombinant hCG (Ovitrelle) and urinary hCG (Pregnyl) were purchased from Merck Serono Europe (Darmstadt, Germany). hCG-h from early pregnancy (pregnancy week 5) and a patient with testicular cancer were purified from urine by several chromatography steps, including a Sepharose column coupled with B152 antibody, as previously reported (Valmu et al., 2006). Informed consent was obtained from all individuals whose urine samples were used for purification of hCG-isoforms and the use of the samples have been approved by the institutional ethics committee at Helsinki University Hospital. Glycans of these hCG-h preparations and significant enrichment of hyperglycosylated hCG, as defined by the presence of core-2 type O-glycan attached to Ser-132, upon B152 purification have been described previously (Valmu et al., 2006). hCG-h from conditioned culture media (day 12) of human embryonic stem cells (hESC line H9; WiCell Research Institute, Madison, WI) differentiated into trophoblast-like cells with FGF2 inhibition and BMP4 activation (Koel et al., 2017) was purified with B152 antibody affinity chromatography. The hCG concentrations (including hCG-h) were determined by a commercial immunofluorometric assay (PerkinElmer, Turku, Finland), and hCGβ, hCGβcf and hyperglycosylated forms of hCG and hCGβ by specific in-house immunoassays calibrated against WHO standards, except for hCG-h assay, which was calibrated with human choriocarcinoma JEG-3 cell derived hCG (Lee et al., 2013; Alfthan et al., 1992; Stenman et al., 2011; Lempiäinen et al., 2008). The limit of detection of all assays was < 2 pmol/L and the intra-assay coefficient of variation < 10% at concentrations above 10 pmol/L.

2.2. Western immunoblotting

hCG or hCGβ (15–80 nmol/L) were denatured by boiling in NuPAGE® LDS Sample Buffer (Life Technologies, Carlsbad, CA) to which 2.4% β-mercaptoethanol and 13 mmol/L dithiothreitol (DTT) were added. Samples of 20 µl were loaded onto SDS-polyacrylamide gel (NuPAGE® Novex® 4–12% Bis-Tris Gel, Life Technologies). After electrophoresis in MES buffer (Life Technologies) the proteins were transferred to polyvinylidene difluoride membranes, blocked overnight at 4 °C with 1% BSA in 50 mmol/L Tris-buffered saline (TBS), pH 7.7, and with 3% milk powder in TBS at room temperature for one hour. The primary antibody against hCGβ (A0231, DAKO, Glostrup, Denmark) in 3% milk powder in TBS was incubated with the membrane for two hours at room temperature. After washing with TBS containing 0.1% Tween, the membrane was incubated with peroxidase-conjugated anti-rabbit IgG (711-035-152, Jackson ImmunoResearch Laboratories, Ely, UK) for one hour at room temperature. After washing, detection was performed with ECL solution (32209, Thermo Fisher Scientific, Rockford, IL).

2.3. LHCGR activation assay

For monitoring LHCGR activation we used Madin-Darby Canine Kidney (MDCK) cells stably expressing human LHCGR and quantified receptor-triggered changes in cyclic adenosine monophosphate (cAMP) levels by a Förster resonance energy transfer (FRET) biosensor (Mazina et al., 2015a, 2015b, 2017). This method allows highly sensitive detection of LHCGR activation, with the limit of detection (*LoD*) and limit of quantitation (*LoQ*) for hCG being 0.96 pmol/L and 2.9 pmol/L, respectively.

The biosensor expression vector mTurq2Δ_Epac(CD,ΔDEP)_td ^{cp173}Ven (H188) (Klarenbeek et al., 2015) was kindly provided by Dr. Kees Jalink from the Netherlands Cancer Institute, and modified to be used in BacMam system as previously described (Mazina et al., 2015a, 2015b, 2017). Briefly, the pcDNA3.1 + expression vector with the H188 gene was cloned into the pFastBac™ 1 vector, and the polyhedrin promoter from the pFastBac™ 1 vector was replaced with the powerful mammalian cytomegalovirus promoter. The resulting construct was used to produce bacmid DNA, which was subsequently transfected into Spodoptera frugiperda Sf9 insect cells to obtain the BacMam virus. The virus was amplified, and the stocks stored at −80 °C. The viral titers were determined experimentally by a cell size-based assay using Sf9 cells (Mazina et al., 2015b). The mammalian MDCK cell line used for

the measurements was a kind gift from Dr Prema Narayan (Southern Illinois University); the initial cell line had been obtained from ATCC (Manassas, VA) (catalog number CRL 2935). The LHCGR-expressing MDCK cells were grown as adherent monolayers at 37 °C and 5% CO_2 in a humidified incubator in Dulbecco's Modified Eagle's Medium and Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 μg/mL amphotericin B.

The assay protocol was adopted from Mazina and coworkers (Mazina et al., 2015a, 2015b, 2017), except that LHCGR-expressing MDCK cells were used instead of COS-7 cells. Briefly, cells were treated with viral stock (multiplicity of infection: 100–300) in 5 mL of growth medium for 3–4 h at 37 °C in a humidified CO_2 incubator. After pre-incubation with the virus, the medium was aspirated, the cells were trypsinized and resuspended in fresh growth medium containing 10 mM sodium butyrate (Sigma-Aldrich, St. Louis, MO) for enhanced protein expression. Next, the cells were seeded onto a transparent 96-well clear-bottom cell culture plate (BioLite 130188, Thermo Fisher Scientific, Rochester, NY) at the density of about 45,000 cells per well (this number varied for different experiments). The transduced cells were cultured for further 21 h at 37 °C for recombinant protein production. On the day of the assay, the growth medium was replaced with 100 μL phosphate-buffered saline (PBS) one hour prior to the experiment.

The dilutions of hormones were performed on transparent 96-well clear-bottom plates (269620, Thermo Fisher Scientific, Denmark) into PBS containing 7.5 μmol/L BSA to reduce non-specific binding. Low-binding pipette tips were used and a new pipette tip was taken for every dilution. The fluorescence intensities were registered prior and after addition of the hormones using either Synergy™ NEO HTS Multi-Mode Microplate Reader (BioTek Instruments Inc., Winooski, VT) with filter-based detection at excitation wavelength of 420/50 nm and simultaneous dual emission at 485/20 and 540/25 nm, or PHERAstar plate reader (BMG LABTECH GmbH, Ortenberg, Germany), with excitation at 427 nm and simultaneous dual emission at 480 and 530 nm. After addition of hormones, readings were taken every two minutes for 70–120 min. The change in FRET ratio was calculated according to the following formula:

$$\Delta FRET = \frac{\frac{YFP_0}{CFP_0} - \frac{YFP}{CFP}}{\frac{YFP_0}{CFP_0}}$$

where CFP_0 and YFP_0 refer to the fluorescence emissions at 480 nm and 530 nm (or 485 and 540 nm) in the given well before and *CFP* or *YFP* after the hormone treatment, respectively.

To establish EC_{50} for each ligand, Δ*FRET* values, calculated for data points taken 60 min after the addition of hormones, were plotted against concentrations of the given ligand. The curves (dose-response with variable slope) were analyzed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

To establish *LoD* and *LoQ* of the assay, Δ*FRET* values, calculated for data points taken 60 min after addition of normal hCG (WHO reference reagent 99/688), were plotted against concentrations of the given ligand. Because fluorescence was measured using different instruments with different gains, normalization of each data set (n = 9) was necessary: the Δ*FRET* value obtained without hCG was defined as 0%, and the Δ*FRET* value obtained for highest concentration hCG was defined as 100%. Next, linear regression was used for fitting of data points representing 0–2.5 pmol/L hCG using GraphPad Prism 6.0. From the pooled normalized data, the slope of linear regression (S) and standard deviation of blank (σ) were calculated. The *LoD* and *LoQ* values of the assay were then calculated according to the following formulae:

$$LoD = 3.3 \cdot \frac{\sigma}{S}$$

$$LoQ = 10 \cdot \frac{\sigma}{S}$$

To establish kinetic parameters for activation of LHCGR pathways by different hormones, the increase of Δ*FRET* was plotted against time after addition of each hormone preparation. For each hormone, one Δ*FRET* vs time curve was chosen per individual measurement. To ensure maximum quality of data (i.e., representing not too fast kinetics yet a sufficient measurement window), the chosen curve usually featured data measured at hormone concentrations above its EC_{50} value, but not hormone concentrations giving maximal activation. Each curve was then fitted to a one-phase association equation using GraphPad Prism 6.0; Y_0 value was fixed at 0. The rate constant obtained for each curve was then divided by the corresponding concentration of ligand; the average of the pooled data for one ligand yielded the value of the apparent association rate constant $k_{on}{}^{app}$. As we had no information on the concentration of LHCGR in the system, but LHCGR was over-expressed, we did not compensate $k_{on}{}^{app}$ values for receptor concentration assuming that the latter was equal in all measurements.

In studies exploring absence of binding of hCGβ to LHCGR, transfected cells were incubated for 2 h with 100 μL of PBS or solutions of hCGβ (99/650) (final concentration of 1800 pmol/L; 7 wells) or hCGβn (99/692) (1200 pmol/L; 7 wells) in PBS. Measurement of biosensor signal prior to or following addition of the samples confirmed that no change in FRET occurred during 2 h incubation with hCGβn. hCGβ induced a signal, which could be attributed to contamination of the preparation with a small amount of hCG as detected by an immunoassay (Table 1). Next, 10 μL of a dilution series of hCG (99/688) was added to both of the aforementioned series, and another 2 h measurement was performed. In the second measurement, EC_{50} values were established for hCG (99/688) after preincubation with hCGβ or hCGβn. The content of hCG in the hCGβ (99/650) sample was taken into account when calculating EC_{50} value for hCG (99/688).

3. Results

Since the hCG may exist in many different forms, like nicked hCG, which is common in hCG preparations isolated from urine and has been reported to lack LHCGR stimulating activity, we characterized the preparations used by specific immunoassays for hCG heterodimer, free hCGβ, hCGβcf and hyperglycosylated hCG/hCGβ (Table 1). The hCG reference contained about 20% of hyperglycosylated hCG and the hCG-h preparations were ~100% hyperglycosylated. Nicked hCG was detected by reducing Western blotting for hCGβ (Fig. 1). While the nicked hCGβ reference (Fig. 1, lane 8) contained only nicked form, the nicked hCG reference (lane 4) appeared to contain a significant amount, about 50%, of non-nicked form. Pregnyl (lanes 1 and *) also contained some nicked hCG/hCGβ. It is noteworthy that recombinant hCG (Ovitrelle, lane 2) showed an additional lower molecular weight band of hCGβ, suggesting the presence of a variant form of hCG.

As expected, hCG activated LHCGR with high efficiency, while hCGβ was devoid of activity or had only residual activity corresponding to the minor hCG contamination in the samples (Table 1, Fig. 2). The EC_{50} value for LHCGR activation by the hCG reference (Table 1, preparation #1) was 3.3 ± 0.6 pmol/L (mean ± SEM, n = 9). All hCG-h preparations (#4, #5 and #6) had lower potency than hCG, the EC_{50} values varying between 7.1 ± 0.5 and 14 ± 3 pmol/L ($P < 0.05$ for all, unpaired *t*-test with Welch's correction, n = 5–11). The maximal stimulation at high concentrations of hCG (#1–3) and hCG-h (#4–6) were very similar ($P > 0.05$ for all hCG-h preparations as compared to hCG). Nicked hCG (#7) had relatively low activity (24 ± 6 pmol/L, $P < 0.05$ as compared to the hCG reference), which was compatible with the presence of non-nicked intact hCG in the preparation (Table 1, Fig. 1). The activities of commercial urinary hCG (Pregnyl, #2) and recombinant hCG (Ovitrelle, #3) were intermediate between those of the hCG reference and hCG-h preparations (for Ovitrelle, $P < 0.05$ as compared to the hCG reference).

The kinetics of LHCGR activation by different hCG preparations was determined by elucidating the apparent on-rate constants ($k_{on}{}^{app}$)

Table 1
hCG and hCGβ preparations used for LHCGR activation studies.

hCG (source[a])	hCG (%)[b]	hCGβ (%)[b]	hCG(β)-h (%)[c]	hCG(β)n (%)[d]	LHCGR activation,[e] EC_{50} (pmol/L)	LHCGR activation,[e] k_{on}^{app} (s^{-1})
1. hCG (99/688)[f]	99.6	0.4	20	–	3.3 ± 0.6 (n = 9)	(1.2 ± 0.2) × 10^8 (n = 6)
2. hCG (Pregnyl)	93.9	6.1	14	< 10	6.1 ± 1.6 (n = 5)	(9.5 ± 1.8) × 10^7 (n = 3)
3. hCG (Ovitrelle)	99.6	0.4	0.01	–	7.5 ± 1.6 (n = 9)*	(8.6 ± 1.6) × 10^7 (n = 5)
4. hCG-h (TCa)	88.8	11.2	~100	–	10 ± 3 (n = 6)*	(3.7 ± 1.7) × 10^7 (n = 3)*
5. hCG-h (PW 5)	87.9	12.1	~100	–	14 ± 3 (n = 5)*	(3.2 ± 0.9) × 10^7 (n = 5)**
6. hCG-h (stem cell)	94.9	5.1	~100	n.a.	7.1 ± 0.5 (n = 11)***	(6.4 ± 0.6) × 10^7 (n = 11)*
7. hCGn (99/642)[f]	94.5	5.5	25	~50	24 ± 6 (n = 5)*	(2.1 ± 0.8) × 10^7 (n = 5)***
8. hCGβ (99/650)[f,g]	0.4	99.6	9.3	–	> 1000 (n = 3)***	n.a.
9. hCGβn (99/692)[f,g]	1.1	98.9	8.9	~100	> 1000 (n = 3)***	n.a.

[a] PW, pregnancy week; TCa, non-seminomatous germ cell tumor of testis.
[b] Percentage of molar amount of intact hCG and free hCGβ in the preparations (these represent both non-hyperglycosylated and hyperglycosylated forms).
[c] The percentage of hyperglycosylated forms of hCG and hCGβ [hCG(β)-h] is indicative as the assay recognizes hCGβ-h with somewhat lower affinity than hCG-h.
[d] The amount of nicked hCG(β) [hCG(β)n] is a rough estimate based on Western immunoblotting (Fig. 1). -, undetectable; n.a., not analyzed.
[e] Activation of LHCGR by different preparations as defined by the concentration needed for half-maximal activation (EC_{50}) and kinetics of the activation (k_{on}^{app}). Data represents mean ± SEM of 3–11 separate assays (all with three replicates). The concentrations used for LHCGR assay are based on hCG concentration (preparations #1–7) or in case of hCGβ (preparations #8–9) on hCGβ concentration.* indicates $P \leq 0.05$,**$P \leq 0.01$ and***$P \leq 0.001$ as compared to WHO international hCG reference reagent (99/688) (i.e., preparation #1). n.a., not analyzed.
[f] The numbers in parenthesis refer to the NIBSC codes of the preparations.
[g] < 1.0% of the immunoreactive hCGβ represents core fragment (hCGβcf) form as determined by a specific immunoassay (Alfthan et al., 1992).

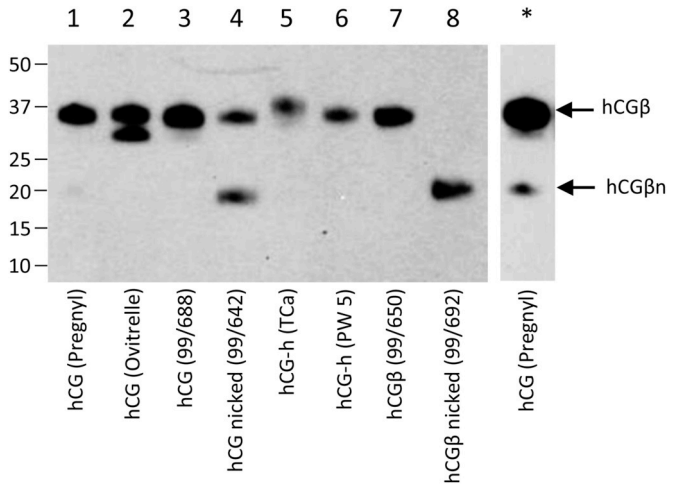

Fig. 1. Detection of nicked hCGβ by Western blotting. *, Note that hCG (Pregnyl) also contained nicked hCG(β), which is well visible only after longer exposure of the blot with film as shown in the right lane.

(Table 1, Fig. 2). Here, we calculated k_{on}^{app} by fitting the obtained ΔFRET curves to the pseudo-first-order exponential equation and normalization of the measured rate factor by total concentration of the hormone in the assay. Because the equilibrium EC_{50} value of a biologically active compound is proportional to its equilibrium dissociation constant K_D, which in turn can be calculated as the ratio of dissociation and association rate constants, substances with low EC_{50} values are expected to feature slow dissociation and fast association kinetics. The comparison of k_{on}^{app} values indeed showed that hCG had the most rapid effect. The effect of hCG-h was slower, and the slowest activation was triggered by nicked hCG, confirming the trends observed for EC_{50} values of different hCG isoforms.

To confirm that hCGβ does not bind to LHCGR, we measured EC_{50} for hCG reference (99/688) after 2 h pre-incubation of cells with elevated concentrations of hCGβ (99/650) (final concentration 1.8 nmol/L) or hCGβn (99/692) (1.2 nmol/L) (Fig. 2). In both cases, the EC_{50} values for hCG reference (99/688) were similar to the EC_{50} value obtained without pre-incubation with hCGβ or hCGβn ($P > 0.05$, two-tailed Student's t-test).

4. Discussion

While hCG appears to have an essential role in early pregnancy, it has remained controversial whether the hyperglycosylated form of hCG, i.e., hCG-h, which is the major isoform of hCG secreted by the placenta during the first 4–5 weeks of pregnancy (Kovalevskaya et al., 1999, 2002a; Stenman et al., 2011), is able to activate LHCGR (Berndt et al., 2013; Crochet et al., 2012; Mazina et al., 2015a). We show here, using highly purified and characterized hCG preparations, that hCG-h activates LHCGR, although with lower potency than non-hyperglycosylated hCG, suggesting functional similarity of these two hCG-isoforms, but also that glycosylation may regulate the activity of hCG during early pregnancy.

The bioactivity of hCG-isoforms has often been studied using cellular systems of non-human species, in which LHCGR is structurally somewhat different from the human receptor (Troppmann et al., 2013). For studies on the differences between hCG-isoforms on LHCGR activation, it is important to use an assay utilizing human LHCGR, like the one used in this study. Using this model we showed that all three studied hCG-h preparations had significantly lower potency for LHCGR activation than the WHO hCG reference, which contained about 20% of hCG-h, the rest representing non-hyperglycosylated form as detected by specific immunoassays. The hyperglycosylated preparations included early pregnancy, testicular cancer and differentiated stem cell-derived hCG-h, indicating that the lower activity is not limited to a certain hCG-h preparation. As shown previously (Catt et al., 1973; Ryan et al., 1988), hCGβ was not able to activate LHCGR. Interestingly, commercial recombinant hCG (Ovitrelle) showed lower activity than the hCG reference and similar trend was observed also for Pregnyl. This may be because of the heterogeneity of Pregnyl, i.e., it contained some hCG-h and nicked hCG, and is known to contain several impurities (Koistinen et al., 2015; Yarram et al., 2004). Ovitrelle seemed to contain two major components, which may represent glycosylation variants. This heterogeneity in SDS electrophoresis has been described also previously (Riccetti et al., 2017) and may explain the reduced activity.

The lower potency of hCG-h for LHCGR activation was supported by our kinetics studies, showing that the effect of hCG-h was slower than that of hCG. Furthermore, we have previously shown that conditioned cell culture medium from human choriocarcinoma JEG-3 cells, which produce hCG-h resembling that from early pregnancy and cancer patients (Valmu et al., 2006), activates LHCGR (Mazina et al., 2015a). When this hCG has been enriched from the media by Concanavalin-A lectin-affinity chromatography, it has been found to be less active than

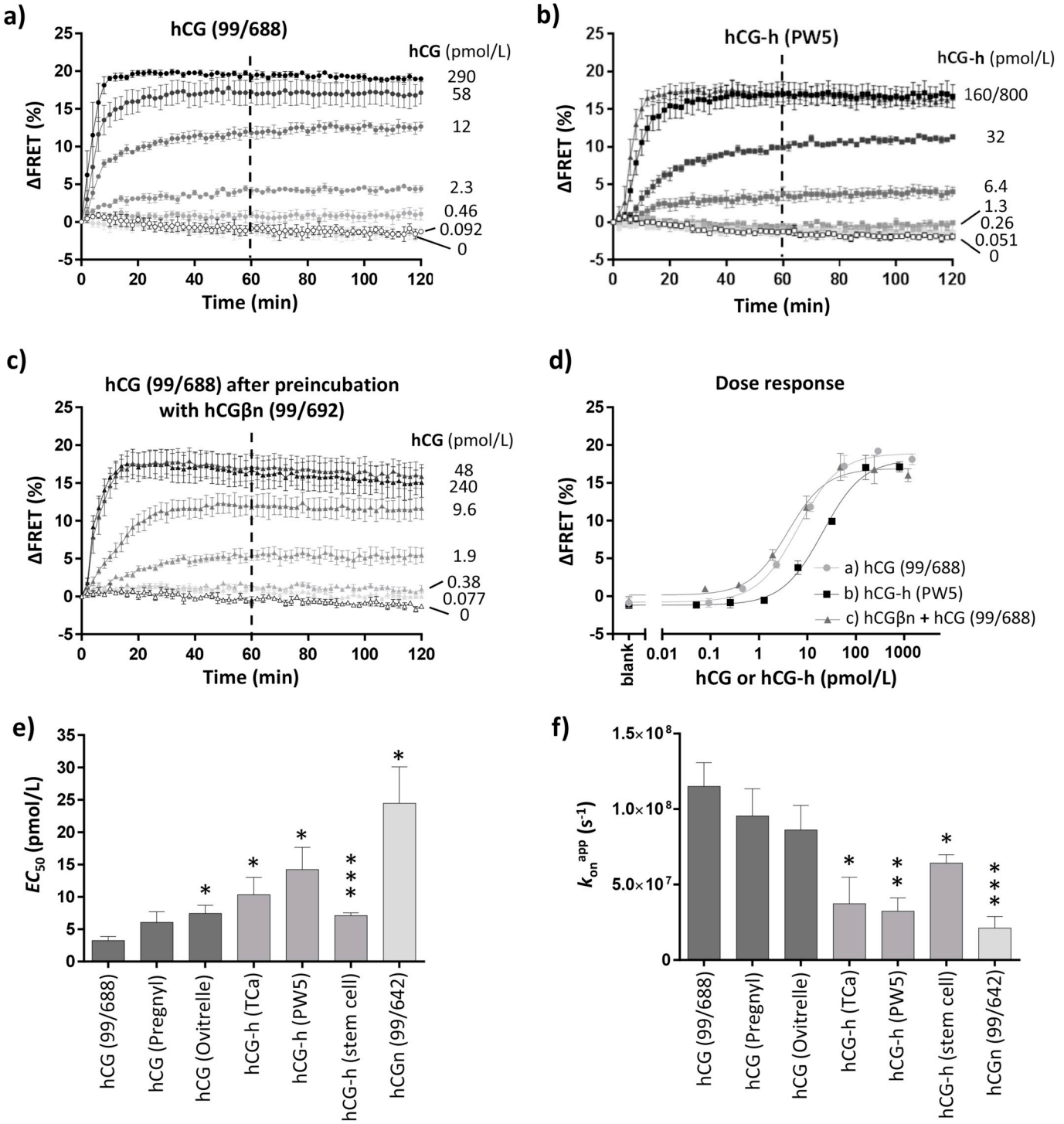

Fig. 2. Activation of LHCGR by different hCG-isoforms. Representative examples of time- and dose-responses in LHCGR activation assay with (a) hCG reference preparation (99/688), (b) hCG-h (purified from pregnancy week 5 urine) and (c) the same preparation as in (a) after 2 h preincubation with hCGβn (99/692). The dotted line indicates 60 min time-point for which dose-response curves are shown in (d). For clarity, the graphs for some of the highest doses, shown in panel d, are not shown in panels a–c. (e) EC_{50}-values for LHCGR activation by different hCG-isoforms and (f) kinetics of the activation. * indicates $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ as compared to WHO international hCG reference reagent (99/688)."

non-hyperglycosylated hCG (Berndt et al., 2013). Contrary to these results, it has been reported that purified hCG-h is unable to activate LHCGR, as determined by the lack of induction of progesterone production in luteinized granulosa cells stimulated with hCG-h (Crochet et al., 2012). These earlier discordant results may be explained by differences between various hCG-h preparations, as many of the studies have been compromised by poor characterization of the preparations used. Especially nicking of the hCG, which is common in hCG isoforms isolated from urine (Bidart et al., 1988; Puisieux et al., 1990; Elliott et al., 1997; Cole et al., 1993), compromises interpretation of the results. We found that the activity of the nicked hCG reference corresponded and, thus, is likely to be explained by the presence of intact non-nicked hCG, which has been estimated to represent 10–15% (Birken et al., 2003b), or even more based on our Western blotting

results, of total hCG in this preparation.

The only apparent difference between hCG-h and hCG is in their glycosylation. In theory, this may affect the tertiary structure of hCG/hCG-h and/or dimer formation with α-subunit, but it is feasible that the glycan(s) are directly responsible for differences in activation of the LHCGR. The variation we observed between different hCG-h preparations may result from variation in glycan structures (Valmu et al., 2006), typical for glycoproteins (Varki, 2017; Koistinen et al., 2003). Previously, the signaling of hCG through LHCGR has been found to be dependent on its glycosylation, e.g., deglycosylated hCG does not induce signaling, although it is able to bind to LHCGR (Sairam, 1989; Richard et al., 2007). While our results show that hCG-h activates LHCGR less efficiently than hCG, the used reporter system is not able to address whether this is due to the steric effects of glycans in hCG-h decreasing the binding to the receptor or due to decreased signal transduction after the hormone has bound to its receptor. Noteworthy, in our experiments excess of hCGβ did not inhibit the response generated by the hCG reference. This suggests that, unlike deglycosylated hCG, hCGβ does not bind to the LHCGR as also reported previously (Catt et al., 1973). Especially, the glycan attached to Asn-52 in hCGα appears to be important for LHCGR activation (Matzuk et al., 1989), but the regulation of hCG activity by glycosylation is complex, and thus other glycans are also likely to play a role. Such a regulation of glycoprotein activity for their cognate receptors has been well established in several systems (Varki, 2017). Interestingly, LH and hCG, both of which signal through LHCGR, have been found to have somewhat different effects downstream of LHCGR activation, including modulation of activity of certain protein kinases and gene expression (Casarini et al., 2012, 2018). Whether hCG and hCG-h are different in this respect (Arey and López, 2011) remains to be studied.

The lower potency of hCG-h to stimulate LHCGR may have (patho) physiological consequences during the first few weeks after the implantation, when hCG-h is the major hCG isoform (Kovalevskaya et al., 1999, 2002a; Stenman et al., 2011). Later on, after 4–5 weeks of pregnancy, when hCG-h is still the dominant hCG isoform, its levels are already significantly increased, which may compensate its lower potency in LHCGR activation in corpus luteum and other tissues. This is supported by our observation that maximal activation of LHCGR was similar with both hCG and hCG-h, although higher concentrations of hCG-h were needed for that. During the first weeks of pregnancy, hCG-h is likely to stimulate trophoblast invasion independently of the LHCGR activation (Lee et al., 2013), but it may also have LHCGR mediated function(s), in addition to corpus luteum, also in uterine and other cells (Ascoli et al., 2002). Interestingly, a recent study reported a woman with an inactive mutant LHCGR who maintained a normal pregnancy after becoming pregnant with ovum donation (Mitri et al., 2014). This suggests that the maintenance of pregnancy through LHCGR activation is not necessary and that during pregnancy the main hCG effect may be mediated by other mechanisms than LHCGR activation. Clinical studies have suggested that low concentrations of hCG-h or low ratio of hCG-h to hCG already on the day of implantation is associated with pregnancy loss (Cole, 2012; Sasaki et al., 2008). However, during that time the levels of hCG-h and hCG are extremely low, making their accurate detection difficult. Contrary to these finding, in IVF treatments it has been found that during the first weeks after embryo transfer the proportion of hCG-h to hCG stayed higher in women with early pregnancy loss, as compared to those with a successful IVF pregnancy (Kovalevskaya et al., 2002b). Later during the first trimester of the pregnancy a reduced proportion of hCG-h to hCG has been found to indicate risk for early onset pre-eclampsia (Keikkala et al., 2013). Although these studies suggest that hCG-h is involved in normal early pregnancy and perhaps also in early onset pregnancy complications, its specific role remains to be established. It is important to note, that the half-life of hCG-h/hCG in circulation and, thereby, the biopotency may also be affected by differences in glycosylation (Mi et al., 2014). So far, the half-life of hCG-h is not known.

We have previously shown that when human embryonic stem cells are differentiated into trophoblast-like cells with FGF2 inhibition and BMP4 activation they produce hCG-h (Koel et al., 2017), which suggests that these differentiated cells are similar to early pregnancy cytotrophoblast cells (Kovalevskaya et al., 2002a). Here we report that the hCG-h produced by these differentiated embryonic stem cells is able to activate LHCGR. Thus, this stem cell model would be suitable for studying the function(s) of trophoblast derived hCG-h.

In conclusion, we showed here that hyperglycosylated hCG is able to activate LHCGR, although with lower potency than hCG. This strongly suggests that the hCG-h is functionally similar to hCG. Whether this explains the reduced proportion of hCG-h to hCG found in patients developing early onset pre-eclampsia or those having early pregnancy loss remains to be determined.

Disclosure summary

None declared.

Acknowledgements

The authors thank Ms. Taina Grönholm and Ms. Annikki Löfhjelm for excellent technical assistance. Funding: This work was supported by the Finnish Cancer Foundation; Sigrid Jusélius Foundation; the Finnish Society of Clinical Chemistry; the Estonian Ministry of Education and Research (IUT34-16 and IUT20-17); Enterprise Estonia (EU48695); the Horizon 2020 innovation program (WIDENLIFE, 692065); European Union FP7 Marie Curie Industry-Academia Partnerships and Pathways funding (IAPP, SARM, EU324509); and the MSCA-RISE-2015 project MOMENDO (691058).

References

Alfthan, H., Haglund, C., Dabek, J., Stenman, U.H., 1992. Concentrations of human choriogonadotropin, its beta-subunit, and the core fragment of the beta-subunit in serum and urine of men and nonpregnant women. Clin. Chem. 38 (10), 1981–1987.

Arey, B.J., López, F.J., 2011. Are circulating gonadotropin isoforms naturally occurring biased agonists? Basic and therapeutic implications. Rev. Endocr. Metab. Disord. 12 (4), 275–288.

Ascoli, M., Fanelli, F., Segaloff, D.L., 2002. The lutropin/choriogonadotropin receptor, a 2002 perspective. Endocr. Rev. 23 (2), 141–174.

Berndt, S., Blacher, S., Munaut, C., Detilleux, J., Perrier d'Hauterive, S., Huhtaniemi, I., Evain-Brion, D., Noël, A., Fournier, T., Foidart, J.M., 2013. Hyperglycosylated human chorionic gonadotropin stimulates angiogenesis through TGF-β receptor activation. Faseb. J. 27 (4), 1309–1321.

Bidart, J.M., Puisieux, A., Troalen, F., Foglietti, M.J., Bohuon, C., Bellet, D., 1988. Characterization of a cleavage product in the human choriogonadotropin beta-subunit. Biochem. Biophys. Res. Commun. 154 (2), 626–632.

Birken, S., Armstrong, E.G., Kolks, M.A., Cole, L.A., Agosto, G.M., Krichevsky, A., Vaitukaitis, J.L., Canfield, R.E., 1988. Structure of the human chorionic gonadotropin beta-subunit fragment from pregnancy urine. Endocrinology 123 (1), 572–583.

Birken, S., Krichevsky, A., O'Connor, J., Schlatterer, J., Cole, L., Kardana, A., Canfield, R., 1999. Development and characterization of antibodies to a nicked and hyperglycosylated form of hCG from a choriocarcinoma patient: generation of antibodies that differentiate between pregnancy hCG and choriocarcinoma hCG. Endocrine 10 (2), 137–144.

Birken, S., Yershova, O., Myers, R.V., Bernard, M.P., Moyle, W., 2003a. Analysis of human choriogonadotropin core 2 o-glycan isoforms. Mol. Cell. Endocrinol. 204 (1–2), 21–30.

Birken, S., Berger, P., Bidart, J.M., Weber, M., Bristow, A., Norman, R., Sturgeon, C., Stenman, U.H., 2003b. Preparation and characterization of new WHO reference reagents for human chorionic gonadotropin and metabolites. Clin. Chem. 49 (1), 144–154.

Casarini, L., Lispi, M., Longobardi, S., Milosa, F., La Marca, A., Tagliasacchi, D., Pignatti, E., Simoni, M., 2012. LH and hCG action on the same receptor results in quantitatively and qualitatively different intracellular signaling. PloS One 7 (10), e46682.

Casarini, L., Santi, D., Brigante, G., Simoni, M., 2018. Two hormones for one receptor: evolution, biochemistry, actions and pathophysiology of LH and hCG. Endocr. Rev. https://doi.org/10.1210/er.2018-00065. ([Epub ahead of print]).

Catt, K.J., Dufau, M.L., Tsuruhara, T., 1973. Absence of intrinsic biological activity in LH and hCG subunits. J. Clin. Endocrinol. Metab. 36 (1), 73–80.

Cole, L.A., 2010. Hyperglycosylated hCG, a review. Placenta 31 (8), 653–664.

Cole, L.A., 2012. Hyperglycosylated hCG and pregnancy failures. J. Reprod. Immunol. 93 (2), 119–122.

Cole, L.A., Butler, S., 2012. Hyperglycosylated hCG, hCGβ and hyperglycosylated hCGβ:

interchangeable cancer promoters. Mol. Cell. Endocrinol. 349 (2), 232–238.
Cole, L.A., Kardana, A., Park, S.Y., Braunstein, G.D., 1993. The deactivation of hCG by nicking and dissociation. J. Clin. Endocrinol. Metab. 76 (3), 704–710.
Crochet, J.R., Shah, A.A., Schomberg, D.W., Price, T.M., 2012. Hyperglycosylated human chorionic gonadotropin does not increase progesterone production by luteinized granulosa cells. J. Clin. Endocrinol. Metab. 97 (9), E1741–E1744.
Elliott, M.M., Kardana, A., Lustbader, J.W., Cole, L.A., 1997. Carbohydrate and Peptide structure of the α- and β-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. Endocrine 7 (1), 15–32.
Evans, J., Salamonsen, L.A., Menkhorst, E., Dimitriadis, E., 2015. Dynamic changes in hyperglycosylated human chorionic gonadotrophin throughout the first trimester of pregnancy and its role in early placentation. Hum. Reprod. 30 (5), 1029–1038.
Fournier, T., Guibourdenche, J., Evain-Brion, D., 2015. Review: hCGs: different sources of production, different glycoforms and functions. Placenta 36 (Suppl. 1), S60–S65.
Handschuh, K., Guibourdenche, J., Tsatsaris, V., Guesnon, M., Laurendeau, I., Evain-Brion, D., Fournier, T., 2007. Human chorionic gonadotropin produced by the invasive trophoblast but not the villous trophoblast promotes cell invasion and is down-regulated by peroxisome proliferator-activated receptor-gamma. Endocrinology 148 (10), 5011–5019.
Hoermann, R., Berger, P., Spoettl, G., Gillesberger, F., Kardana, A., Cole, L.A., Mann, K., 1994. Immunological recognition and clinical significance of nicked human chorionic gonadotropin in testicular cancer. Clin. Chem. 40 (12), 2306–2312.
Keikkala, E., Vuorela, P., Laivuori, H., Romppanen, J., Heinonen, S., Stenman, U.H., 2013. First trimester hyperglycosylated human chorionic gonadotrophin in serum - a marker of early-onset preeclampsia. Placenta 34 (11), 1059–1065.
Klarenbeek, J., Goedhart, J., van Batenburg, A., Groenewald, D., Jalink, K., 2015. Fourth-Generation Epac-based FRET sensors for cAMP feature exceptional brightness, photostability and dynamic range: characterization of dedicated sensors for FLIM, for ratiometry and with high affinity. PloS One 10 (4), e0122513.
Koel, M., Võsa, U., Krjutškov, K., Einarsdottir, E., Kere, J., Tapanainen, J., Katayama, S., Ingerpuu, S., Jaks, V., Stenman, U.H., Lundin, K., Tuuri, T., Salumets, A., 2017. Optimizing bone morphogenic protein 4-mediated human embryonic stem cell differentiation into trophoblast-like cells using fibroblast growth factor 2 and transforming growth factor-β/activin/nodal signaling inhibition. Reprod. Biomed. Online 35 (3), 253–263.
Koistinen, H., Easton, R.L., Chiu, P.C., Chalabi, S., Halttunen, M., Dell, A., Morris, H.R., Yeung, W.S., Seppala, M., Koistinen, R., 2003. Differences in glycosylation and sperm-egg binding inhibition of pregnancy-related glycodelin. Biol. Reprod. 69 (5), 1545–1551.
Koistinen, H., Hautala, L., Koli, K., Stenman, U.H., 2015. Absence of TGF-β receptor activation by highly purified hCG preparations. Mol. Endocrinol. 29 (12), 1787–1791.
Kovalevskaya, G., Birken, S., Kakuma, T., O'Connor, J.F., 1999. Early pregnancy human chorionic gonadotropin (hCG) isoforms measured by an immunometric assay for choriocarcinoma-like hCG. J. Endocrinol. 161 (1), 99–106.
Kovalevskaya, G., Genbacev, O., Fisher, S.J., Caceres, E., O'Connor, J.F., 2002a. Trophoblast origin of hCG isoforms: cytotrophoblasts are the primary source of choriocarcinoma-like hCG. Mol. Cell. Endocrinol. 194 (1–2), 147–155.
Kovalevskaya, G., Birken, S., Kakuma, T., Ozaki, N., Sauer, M., Lindheim, S., Cohen, M., Kelly, A., Schlatterer, J., O'Connor, J.F., 2002b. Differential expression of human chorionic gonadotropin (hCG) glycosylation isoforms in failing and continuing pregnancies: preliminary characterization of the hyperglycosylated hCG epitope. J. Endocrinol. 172 (3), 497–506.
Lee, C.L., Chiu, P.C.N., Hautala, L., Salo, T., Yeung, W.S.B., Stenman, U.H., Koistinen, H., 2013. Human chorionic gonadotropin and its free beta-subunit stimulate trophoblast invasion independent of LH/hCG receptor. Mol. Cell. Endocrinol. 375 (1–2), 43–52.
Lempiäinen, A., Stenman, U.H., Blomqvist, C., Hotakainen, K., 2008. Free β-subunit of human chorionic gonadotropin in serum is a diagnostically sensitive marker of seminomatous testicular cancer. Clin. Chem. 54 (11), 1840–1843.
Lempiäinen, A., Hotakainen, K., Blomqvist, C., Alfthan, H., Stenman, U.H., 2012. Hyperglycosylated human chorionic gonadotropin in serum of testicular cancer patients. Clin. Chem. 58 (7), 1123–1129.
Matzuk, M.M., Keene, J.L., Boime, I., 1989. Site specificity of the chorionic gonadotropin N-linked oligosaccharides in signal transduction. J. Biol. Chem. 264 (5), 2409–2414.
Mazina, O., Luik, T., Kopanchuk, S., Salumets, A., Rinken, A., 2015a. Characterization of the biological activities of human luteinizing hormone and chorionic gonadotropin by a förster resonance energy transfer-based biosensor assay. Anal. Lett. 48 (17), 2799–2809.
Mazina, O., Allikalt, A., Heinloo, A., Reinart-Okugbeni, R., Kopanchuk, S., Rinken, A., 2015b. cAMP assay for GPCR ligand characterization: application of BacMam expression system. In: Prazeres, D., Martins, S. (Eds.), G Protein-coupled Receptor Screening Assays. Methods in Molecular Biology. vol. 1272. Springer New York, pp. 65–77.
Mazina, O., Allikalt, A., Tapanainen, J.S., Salumets, A., Rinken, A., 2017. Determination of biological activity of gonadotropins hCG and FSH by Förster resonance energy transfer based biosensors. Sci. Rep. 7, 42219.
Mi, Y., Lin, A., Fiete, D., Steirer, L., Baenziger, J.U., 2014. Modulation of mannose and asialoglycoprotein receptor expression determines glycoprotein hormone half-life at critical points in the reproductive cycle. J. Biol. Chem. 289 (17), 12157–12167.
Mitri, F., Bentov, Y., Behan, L.A., Esfandiari, N., Casper, R.F., 2014. A novel compound heterozygous mutation of the luteinizing hormone receptor -implications for fertility. J. Assist. Reprod. Genet. 31 (7), 787–794.
Nisula, B.C., Blithe, D.L., Akar, A., Lefort, G., Wehmann, R.E., 1989. Metabolic fate of human choriogonadotropin. J. Steroid Biochem. 33 (4B), 733–737.
Pierce, J.G., Parsons, T.F., 1981. Glycoprotein hormones: structure and function. Annu. Rev. Biochem. 50, 465–495.
Puisieux, A., Bellet, D., Troalen, F., Razafindratsita, A., Lhomme, C., Bohuon, C., Bidart, J.M., 1990. Occurrence of fragmentation of free and combined forms of the beta-subunit of human chorionic gonadotropin. Endocrinology 126 (2), 687–694.
Riccetti, L., Klett, D., Ayoub, M.A., Boulo, T., Pignatti, E., Tagliavini, S., Varani, M., Trenti, T., Nicoli, A., Capodanno, F., La Sala, G.B., Reiter, E., Simoni, M., Casarini, L., 2017. Heterogeneous hCG and hMG commercial preparations result in different intracellular signaling but induce a similar long-term progesterone response in vitro. Mol. Hum. Reprod. 23 (10), 685–697.
Richard, C.A., Creinin, M.D., Kubik, C.J., DeLoia, J.A., 2007. Enzymatic removal of asparagine-linked carbohydrate chains from heterodimer human chorionic gonadotrophin and effect on bioactivity. Reprod. Fertil. Dev. 19 (8), 933–946.
Ryan, R.J., Charlesworth, M.C., McCormick, D.J., Milius, R.P., Keutmann, H.T., 1988. The glycoprotein hormones: recent studies of structure-function relationships. Faseb. J. 2 (11), 2661–2669.
Sairam, M.R., 1989. Role of carbohydrates in glycoprotein hormone signal transduction. Faseb. J. 3 (8), 1915–1926.
Sakakibara, R., Miyazaki, S., Ishiguro, M., 1990. A nicked beta-subunit of human chorionic gonadotropin purified from pregnancy urine. J. Biochem. 107 (6), 858–862.
Sasaki, Y., Ladner, D.G., Cole, L.A., 2008. Hyperglycosylated human chorionic gonadotropin and the source of pregnancy failures. Fertil. Steril. 89 (6), 1781–1786.
Stenman, U.H., Alfthan, H., 2013. Determination of human chorionic gonadotropin. Best Pract. Res. Clin. Endocrinol. Metabol. 27 (6), 783–793.
Stenman, U.H., Alfthan, H., Hotakainen, K., 2004. Human chorionic gonadotropin in cancer. Clin. Biochem. 37 (7), 549–561.
Stenman, U.H., Tiitinen, A., Alfthan, H., Valmu, L., 2006. The classification, functions and clinical use of different isoforms of HCG. Hum. Reprod. Update 12 (6), 769–784.
Stenman, U.H., Birken, S., Lempiäinen, A., Hotakainen, K., Alfthan, H., 2011. Elimination of complement interference in immunoassay of hyperglycosylated human chorionic gonadotropin. Clin. Chem. 57 (7), 1075–1077.
Troppmann, B., Kleinau, G., Krause, G., Gromoll, J., 2013. Structural and functional plasticity of the luteinizing hormone/choriogonadotrophin receptor. Hum. Reprod. Update 19 (5), 583–602.
Valmu, L., Alfthan, H., Hotakainen, K., Birken, S., Stenman, U.H., 2006. Site-specific glycan analysis of human chorionic gonadotropin beta-subunit from malignancies and pregnancy by liquid chromatography-electrospray mass spectrometry. Glycobiology 16 (12), 1207–1218.
Varki, A., 2017. Biological roles of glycans. Glycobiology 27 (1), 3–49.
Yarram, S.J., Jenkins, J., Cole, L.A., Brown, N.L., Sandy, J.R., Mansell, J.P., 2004. Epidermal growth factor contamination and concentrations of intact human chorionic gonadotropin in commercial preparations. Fertil. Steril. 82 (1), 232–233.