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INFLUENCE OF CYCLIC NUCLEOTIDES ON THE PROCESSING OF THE CARBOHYDRATE PART OF THE α-SUBUNIT OF HUMAN CHORIOGONADOTROPIN BY FIRST TRIMESTER HUMAN PLACENTA TISSUE

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In pulse-chase experiments ([^{35}S]Met as radioactive label) 4 intracellular forms of the α-subunit (apparent molecular weights of 11, 16.5, 19.5, and 23.4 kDa) were observed whereas almost no label was incorporated into the β-subunit. The 23.4 kDa form was secreted as free α-subunit, the others were precursors of the α-subunit contained in secreted human choriogonadotropin. The rate-limiting step seemed to be the processing of the 19.5 kDa precursor by α-mannosidase II. 8-bromo-cAMP increased the total amount of intracellular forms of the α-subunit and accelerated significantly the velocity of all glycosylation steps. It seemed to cause a higher efficacy of the α-mannosidase II reaction. In the presence of 8-bromo-cAMP intracellular as well as extracellular α-subunits showed a higher sialic acid content.

Human choriogonadotropin which is synthesized by the placenta of the first trimester of pregnancy is composed of two dissimilar, noncovalently linked subunits, α and β (1, 2). It is a glycoprotein with a carbohydrate moiety of about 30%. Two N-linked carbohydrate parts of the N-acetyllactosamine type (without fucose in the case of the α-subunit) are connected with each subunit (3). In addition, the β-subunit has 4 O-linked carbohydrate parts attached to its C-terminal region (4). Highly purified urinary hCG shows microheterogeneity due to a different content in N-acetylneuraminic acid (5, 6). In an earlier study (7) we have shown that, at day 3 of cultures of placenta tissue, 8-bromo-cAMP caused an increase of the hCG secretion rate, shifted the band pattern of hCG in isoelectric focusing in favor of the more acidic subpopulations, and increased significantly the receptor binding activity as well as the biological activity of the hormone. It is known that cAMP stimulates hCG biosynthesis by placenta tissue (8-11), however,

Abbreviations: Endo H, endo-β-N-acetylglucosaminidase H; hCG, human choriogonadotropin.

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it remained unclear how this is effected. One mechanism might be a direct or indirect control of the expression of the hCG subunit genes since it was shown that millimolar concentrations of 8-bromo-cAMP stimulated transcription rate 15-30 fold (12). Our earlier results suggested that 8-bromo-cAMP might also influence the glycosylation (7). In our present study we investigate the processing of the carbohydrate part of the α-subunit. Evidence is provided that cAMP accelerates early as well as terminal steps of glycosylation of the hCG-α-subunit which results in the formation of hCG species containing an increased sialic acid content.

MATERIALS AND METHODS

Placenta Tissue Culture.

Placenta tissue (from legal abortions; 8th-10th week of gestation) was minced into pieces of 20-30 mg wet weight and cultured in 1.5 ml tissue culture medium 199 per piece of tissue in an atmosphere of 95 % air and 5 % CO_2 (medium changed daily). The tissue culture medium 199 with Hanks' balanced salt solution contained per litre: 116 mg penicillin G sodium salt, 200 mg streptomycin sulphate, 2.5 mg amphotericin B and 10 % newborn calf serum. Cyclic nucleotides were added in the following concentrations: cAMP (3 mM), 8-bromo-cAMP (0.5 mM), 8-bromo-cGMP (0.5 mM), IBMX (0.1 mM), IBMX (0.1 mM) + cAMP (1 mM), and IBMX (0.1 mM) + cGMP (0.1 mM).

Pulse-chase experiments.

At day 3 of culture, tissue was preincubated (1 h) in Met-free medium. A pulse of 7.4 MBq [^{35}S]Met/ml (49.2 TBq/mmol; Amersham-Buchler, Braunschweig, F.R.G.) was applied for 30 min. The radioactive label was chased for 5, 20, 60 or 120 min, respectively. Tissue culture media were collected and stored (-70°C). Placenta tissue was homogenized in 100 µl homogenization buffer (see below) by sonication at 0°C. The homogenization buffer contained per litre: 10 mmol sodium phosphate, 154 mmol NaCl, N-tosyl-L-lysyl-chloromethane hydrochloride (0.1 mmol), Trasylol (100,000 IU), and leupeptin (1 mmol). After sonication the samples were centrifuged at 130,000 g for 5 min (Beckman Airfuge). The supernatants were stored at -70°C. In some experiments a mixture of tritiated amino acids (L-[4.5-^{3}H]leucine, L-[4.5-^{3}H]lysine, L-[2.3.4.5.6-^{3}H]phenylalanine, L-[2.3.4.5-^{3}H]proline, L-[2.3.5.6-^{3}H]tyrosine; Amersham-Buchler, Braunschweig, F.R.G.) was used for labeling (2 h preincubation in culture medium devoid of these amino acids; labeling (2.83 MBq/ml) within 4 h; further processed as described above ommitting the chase procedure).

Immunoadsorption.

Antisera against purified hCG and the free α-subunit were prepared in goats as previously described ((13); cross-reactivities determined by radioimmunoassays: anti hCG: 1.6% with the α-, 0.3% with the β-subunit; anti free α-subunit: 0.2% with hCG; 0.2% with the β-subunit (Layer, Mayer and Merz, unpublished)). Purified IgGs (obtained from 5 ml of antiserum) were coupled to (20 ml packed) Sepharose CL-4B with the cyanogen bromide method (14). 40 µl of the immunosorbent were filled into small columns (disposable pipette tips, bed volume 5 x 2 mm). Samples to be immunoadsorbed were diluted with homogenization buffer to a final volume of 200 µl and applied to the columns. The effluent was reapplied once more. Columns were washed with 2 ml homogenization buffer, 0.7 ml of 0.01 M sodium citrate buffer (pH 4.0), and centrifuged for 1 minute at 10,000 g. Finally, hCG and the hCG-α-subunit, respectively, were released by incubation in 0.01 M citrate buffer containing 6 M urea (pH 4.0) at 80°C for 5 min, followed by centrifugation of the columns (1 min at 10,000 g).

Digestion with Neuraminidase and Endo H.

Carbohydrate processing was investigated by means of digestibility with Endo H (E.C. 3.2.1.96 from Streptomyces plicatus, Boehringer, Mannheim, F.R.G.) and neuraminidase (E.C. 3.2.1.18 from Vibrio cholerae; Behringwerke, Marburg, F.R.G.). Prior to digestion, the samples were desalted (Sephadex G 25 microcolumns, 5 x 110

mm). Digestion with Endo H was carried out at pH 5.5 in 0.1 M sodium citrate buffer (1 μU/1000 cpm, 37°C, 3 and 6 h, respectively. The digestion with neuraminidase was performed at 37°C for 3 h (10 μU/1000 cpm) in a buffer containing per litre: 33.3 mmol sodium acetate, 103 mmol sodium chloride and 6 mmol calcium chloride (pH 5.5).

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis and Isoelectric Focusing.

Electrophoresis (500-1,000 cpm applied per lane) was performed as described by Lämmli (15) (polyacrylamide gradient 7-20 % T, 2.6 % C; gel size 85 x 85 x 0.75 mm). Thereafter, the proteins were precipitated with 20 % (w/v) trichloroacetic acid. Radioactivity was detected by fluorography (16) using Kodak X-O-mat X-ray film. For quantitative evaluations, the X-ray films were scanned 3 times per lane by means of a 2202 Ultroscan laser densitometer (LKB, Bromma, Sweden). Isoelectric focusing was performed in the presence of 6 M urea as previously described (17). Prior to the isoelectric focusing the samples were desalted (Sephadex G 25 m column, 5x110 mm), lyophilized and dissolved in 6 M urea (pH 4.0). After the separation the proteins were precipitated with 20% trichloroacetic acid. [^{35}S] Met-labeled proteins were detecteded by autoradiography on Kodak X-O-mat X-ray film.

RESULTS AND DISCUSSION

Processing of the α-subunit.

In control cultures (no addition of cyclic nucleotides) four intracellular forms of the α-subunit with apparent molecular weights of 11 kDa, 16.5 kDa, 19.5 kDa, and 23.4 kDa (Fig. 1: A lane 3; B lanes 1-4) were observed (isolated by immunoadsorption with anti free α-subunit-Sepharose). Except the 23.4 kDa species, all forms seemed to be precursors of the α-subunit part of secreted hCG. The 11 kDa band was considered to be a carbohydrate-free precursor since it was not digested by Endo H and neuraminidase. Furthermore, it was observed in the case of in vitro translation experiments and in tunicamycin-treated cultures (not shown). It was very rapidly converted into the 16.5 kDa precursor. This might suggest that N-glycosylation of the α-subunit possibly does not take place cotranslationally. The 16.5 kDa form seemed to contain a carbohydrate residue of the high mannose type since it was converted into a 11 kDa form by digestion with Endo H (Fig. 2) and was neuraminidase insensitive. The 19.5 kDa form was partially sialylated and was completely transformed into 16.5 kDa and 11 kDa forms by treatment with Endo H (Fig. 2, lane 7). This indicates that the 19.5 kDa form probably contained mannose-rich carbohydrate residues. In accordance with Hussa (18) we could detect intracellularly neither the mature α-subunit as contained in hCG (apparent molecular weight 20.6 kDa, Fig. 1 A) nor hCG itself while others described the accumulation of the mature α-subunit in the tissue (19). Our results suggest that the formation of the mature α-subunit from the 19.5 kDa precursor is immediately followed by the assembly with the β-subunit and the secretion of the hCG molecule. Therefore, the completion of the carbohydrate part of the α-subunit is possibly a signal for subunit assembly and secretion of the hormone. The influence of the carbohydrate moieties on the ability of the α-subunit to recombine with the β-subunit as described by Strickland and Pierce (20) seem to support this

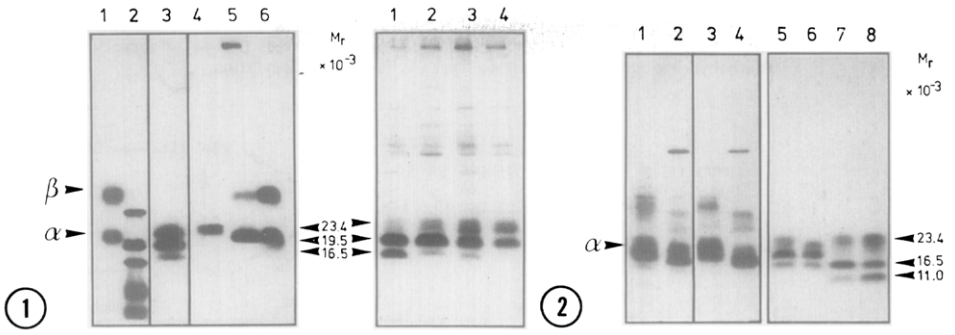

Fig. 1: **Incorporation of [^{35}S]Met into hCG α-subunits synthesized by human first trimester placenta.** Part A: Intracellular and secreted forms. Lanes 1,6: mixture of isolated ^{125}I-labeled hCG subunits. Lane 2: ^{14}C-labeled standard proteins, from top to bottom: carbonic anhydrase (30.0 kDa), soy bean trypsin inhibitor (21.5 kDa), cytochrome C (12.5 kDa), aprotinin (6.5 kDa), insulin B chain (3.4 kDa). Lane 3: intracellular forms of the α-subunit (60 min chase). Lane 4: secreted free α-subunit. Lane 5: secreted hCG. Part B: Processing of the intracellular α-subunits in a pulse-chase experiment (30 min pulse), chase intervals of 5, 20, 60, and 120 min (lanes 1-4). A 11 kDa band which was visible at 5 min chase on the original fluorograph was too faint for reproduction on the photograph.

Fig. 2: **Sensitivity of intracellular α-subunit forms to endo-β-N-acetyl-glucosaminidase H and neuraminidase.** α-subunit forms were digested with neuraminidase (lanes 2,4,6) and endo-β-N-acetylglucosaminidase H (3 h, lane 7; 6 h, lane 8). The position of the α-subunit contained in hCG is marked by α. Lanes 1-4: labeling (4 h) with a mixture of tritiated amino acids (Leu, Lys, Phe, Pro, Tyr) in the absence (lanes 1,2) and presence of 0.5 mM 8-bromo-cAMP (lanes 3,4). Lanes 5-8: pulse-chase labeling with [^{35}S]Met (30 min pulse, 60 min chase) in untreated control cultures.

hypothesis. The 23.4 kDa form which appeared in the later chase interval was sialylated and contained no Endo H sensitive structures (Fig. 2). It was described that α-subunits which seem to correspond to the 19.5 kDa and the 23.4 kDa forms characterized here did not associate with the purified hCG β-subunit (21). The α-subunit part of secreted hCG showed an apparent molecular weight of 20.6 kDa and was distinctly labeled in contrast to the β-subunit (Fig. 1 A lane 5). In addition to hCG a strongly labeled free α-subunit was secreted (lane 4). It showed the same properties as the intracellular 23.4 kDa form. This might indicate that the fraction of the α-subunit which had not been associated with the β-subunit was submitted to further glycosylation and was secreted as the free 23.4 kDa α-subunit. A free α-subunit with a higher apparent molecular weight than the α-subunit of hCG, a higher sialic acid content and a different carbohydrate structure was also found by others (19, 22-28). It was suggested that the surplus in glycosylation might prevent the assembly with the β-subunit for steric reasons (25, 29).

The results presented here suggest a model for the processing of the N-linked carbohydrate residues of the α-subunit and the subunit assembly as summarized in Fig. 3. The α-mannosidase II step (conversion of GlcNAc(Man)$_5$(GlcNAc)$_2$-Asn

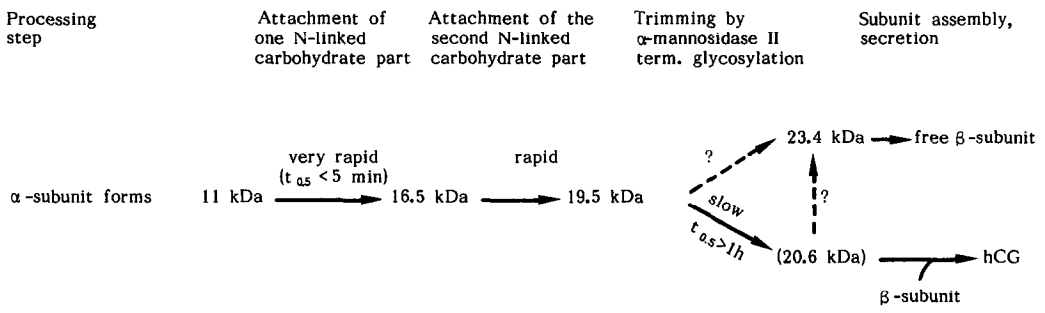

Fig. 3: **Hypothetical model for the post-translational processing of the hCG α-subunit.**

into GlcNAc(Man)$_3$(GlcNAc)$_2$-Asn) is probably rate-limiting for this pathway. The accumulation of the 19.5 kDa form at this step in part caused the promotion of terminal glycosylation (inclusive the addition of N-acetylneuraminic acid) and thus yielding a 19.5 kDa form of the hybrid type (not shown). Other investigators described that an earlier reaction (catalyzed by α-mannosidase I) as the rate-limiting step (19, 22). The differences between these findings and our observations cannot be explained at present.

Influence of Cyclic Nucleotides and IBMX

CGMP and 8-bromo-cGMP showed no distinct effect at day 3 of culture which is in accordance with our earlier studies (7). In contrast, cAMP, cAMP in combination with IBMX, and especially 8-bromo-cAMP, caused an increase of the intracellular concentrations as well as a significant ($p < 0.05$) acceleration of the initial formation rates of the α-subunit forms which was evident from the quantitative evaluation of the fluorographs by densitometry (not shown). 8-bromo-cAMP seemed to cause a greater supply with untrimmed precursor than could be processed at the level of the α-mannosidase II reaction since the special form of the 19.5 kDa precursor probably containing a carbohydrate part of the hybride type was accumulated. Whereas the α-subunit of secreted hCG in control cultures was in part sensitive to digestion with Endo H (Fig. 4, lanes 2, 5) this was not observed in the presence of 8-bromo-cAMP (α-subunit contained in hCG was completely Endo H resistant). This seems to indicate that the terminal glycosylation proceeded more efficiently in the presence of 8-bromo-cAMP. Furthermore, in the medium of cultures treated with 8-bromo-cAMP two additional acidic variants of the free α-subunit were observed or were synthesized in considerable higher concentrations than in the control cultures (Fig. 5 F, G). The same was also observed with respect to the intracellular α-subunits (lanes D, E). The most basic form of the intracellular α-subunit, which showed the isoelectric point of the asialo α-subunit observed in control cultures (lane D), was missing in the cultures treated with 8-bromo-cAMP (lane E). The respective band was shifted towards the anode which indicates an increased content of sialic acid (digestible with neuraminidase). Moreover, hCG

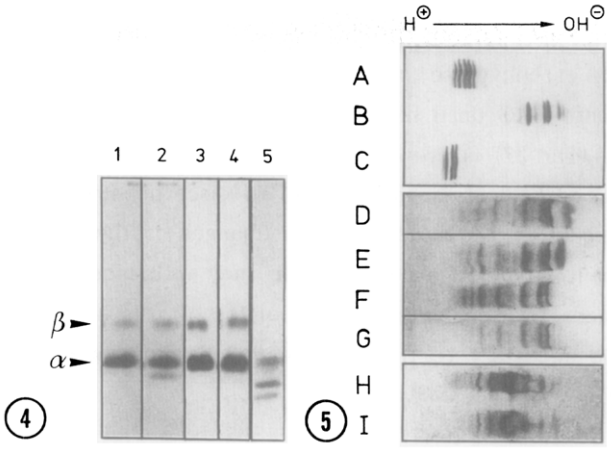

Fig 4: **Sensitivity of the α-subunit of secreted hCG to endo-β-N-acetylglucosaminidase H.** HCG was labeled under control conditions (lanes 1,2,5) and in the presence of 0.5 mM 8-bromo-cAMP (lanes 3,4) with a mixture of tritiated amino acids (Leu, Lys, Phe, Pro, Tyr; 4 h; lanes 1-4) or with [^{35}S]Met in a pulse-chase experiment (30 min pulse, 60 min chase; lane 5). Lanes 2,4,5: digestion with endo-β-N-acetylglucosaminidase H for 6 h. HCG subunits are marked by α and β.

Fig. 5: **Influence of 8-bromo-cAMP on the microheterogeneity of hCG and its α-subunit.** Microheterogeneity of purified urinary hCG (A), the isolated α-subunit (B), and the isolated β-subunit (C). Intracellular forms of the α-subunit synthesized under control conditions (D) and in the presence of 0.5 mM 8-bromo-cAMP (E). Secreted free α-subunit, cultures treated with 8-bromo-cAMP (F) and control cultures (G), respectively. α-subunit contained in hCG secreted in the presence of 8-bromo-cAMP (H) and in untreated controls (I). Proteins (A-C) stained with Coomassie Blue R-250, radioactivity (30 min pulse with [^{35}S]Met, 60 min chase) visualized by autoradiography (D-I).

synthesized in the presence of 8-bromo-cAMP showed in addition molecules with lower isoelectric points in comparison to the hCG of control cultures (lanes H, I). The most basic intracellular forms were found neither in the free α-subunit isolated from the culture medium nor in the secreted hormone (compare Fig. 5 lanes D-E, F-G and H-I). The α-subunit of hCG secreted in 8-bromo-cAMP-treated cultures showed subspecies with lower isoelectric points than the α-subunit of purified urinary hCG.

At present, our data provide no evidence of a mechanism which might explain the cAMP effect on the glycosylation of the hCG-α-subunit. Recently, Camilli et al. (30) described an association of cAMP receptor proteins (cAMP binding subunits of protein kinases II) with microtubules and with structures close to the Golgi apparatus in brain tissue. It was suggested that cAMP may indirectly influence processes in the (trans) Golgi e.g. by regulating the vesicle transport. This may explain some effects of cyclic nucleotides on the hCG-biosynthesis in placenta tissue, however, it remains to be shown that this model can be applied to the placenta. The effect of 8-bromo-cAMP on the terminal glycosylation (including sialylation (Fig. 5)) seems not to fit into the model of the presence of 2 biantennary carbohydrate parts of the complex type as described for the α-subunit

contained in hCG (3), especially in the case of the 23.4 kDa α-subunit. This may be a hint on a different carbohydrate structure. Triantennary carbohydrate chains were described only in tumor hCG until now (31). O-glycosylation may also take place since a suitable site (Thr 37) is present in the α -subunit (29, 32). On the basis of the results presented here it is not possible to discern between these two alternatives with respect to the cAMP-induced changes of the glycosylation of hCG-α-subunits. The present studies show that cAMP may influence glycosylation of hCG which has consequences for the biological properties of this hormone. Further investigations will be necessary to find out which individual processes of glycosylation are affected by cAMP.

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