HUMAN PROLACTIN AND ITS ANTAGONISTS

Soares, C.R.J.; Okazaki, K.; Ueda, E.K.; Gomide, F.I.C.; Heller, S.R.; Arkaten, R.R.; Oliveira, J.E.; Sousa, J.M.; Ribela, M.T.C.P.; Bartolini, P.

Centro de Biologia Molecular - IPEN/CNEN-SP

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This hormone was synthesized in our laboratory by DNA recombinant techniques, in both bacterial and mammalian (CHO) cells. Human prolactin is widely used for "in vitro" diagnostic applications but many possible therapeutic uses have also been reported, such as the stimulation of immune response and hematopoietic recovery of AIDS patients. Recently, the notion that hPRL acts as a survival growth factor in the mammary gland and is directly involved in breast cancer development has revitalized the efforts in searching for a hPRL antagonists that may inhibit this effect. Our laboratory, the first in absolute to obtain authentic human prolactin in bacterial periplasmic space, is developing new bacterial expression vectors utilizing a promoter activated by temperature, to express hPRL as well as two antagonists of this hormone that can inhibit the proliferation of breast and prostate cancer cells. A reversed-phase high-performance liquid chromatography for the analysis of human prolactin in bacterial periplasmic space and in purified preparations was developed, which provided a valuable tool for comparing the hydrophobicity of glycosylated and non-glycosylated prolactin molecules obtained from several different species and of different preparations of native or biosynthetic human prolactin. The synthesis, purification and characterization of G129R-hPRL and S179D-hPRL, the two better studied antagonists of human prolactin (hPRL), is described in a manuscript ready for publication. Both of these have been expressed for the first time, in their authentic form, by a stable CHO cell line, at secretion levels of 7.7 and 4.3 µg/million cells/day, respectively. Previous studies had shown that these hPRL analogs, when produced in bacterial cytoplasm, consistently contained misfolded forms and multimers that may vary their bioactivity according to the denaturation, refolding and purification conditions and, obviously, have an N-terminal extra methionine. An extensive physico-chemical characterization was carried out after a practical two- step purification process and included SDS-PAGE and Western Blotting analysis, matrix-assisted laser desorption ionization time-of-flight mass spectral (MALDI-TOF-MS) analysis, high-performance size-exclusion chromatography (HPSEC) and reversed-phase high-performance liquid chromatography (RP-HPLC). This last technique revealed a considerable difference in hydrophobicity simply due to a single amino acid substitution, with S179D-hPRL less (t_{RR}=0.85±0.01) and G129R-hPRL more (t_{RR}=1.10±0.013) hydrophobic than hPRL. Where t_{RR} is the relative retention time. The biological characterization was based on a novel, ultra-sensitive (minimal detectable dose: 0.04 ng of hPRL/mL) proliferation assay based on a pro-B murine cell line (Ba/F03) transfected with the hPRL receptor gene and grown in the presence of a very low hPRL concentration: the Ba/F3-LLP assay. On the basis of this assay the relative agonistic activity of these two products, determined against a hPRL international standard in four independent assays, was 0.053 for S179D-hPRL and 0.00072 for G129R-hPRL. We believe that the present synthesis and characterization can be extremely helpful for studies of these two proteins, which have been reported to antagonize tumor-growth promoting effects of hPRL in vivo on the basis of animal models of breast and prostate cancer. Unexpectedly, antiangiogenic activity, capable of inhibiting tumor neovascularization, has been detected in hPRL-S179D, thanks to a research work carried out in collaboration with the University of California at Riverside. Our laboratory also participated of the World Health Organization Collaborative Study to assess the suitability of recombinant human prolactin as an International Standard. Analysis including “in vitro” Nb2 bioassay, radioimmunoassay and physico-chemical studies by high performance size exclusion chromatography, reversed-phase high performance liquid chromatography and SDS-PAGE were carried out and included in the study.

HUMAN THYROID-STIMULATING HORMONE (hTSH): SYNTHESIS, PURIFICATION AND CHARACTERIZATION

Ribela, M.T.C.P.; Oliveira, J.E.; Peroni, C.N.; Okazaki, K.; Soares, C.R.J.; Sousa, J.M.; Bartolini, P.

Centro de Biologia Molecular - IPEN/CNEN-SP

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This hormone is not yet produced in Brazil, being its availability very important for *in vivo* use in the diagnosis and therapy of thyroid carcinoma, via stimulation of radioiodine uptake, and *in vitro* use as immunoassay reagent. A high-level expression of hTSH in Chinese hamster ovary cells (CHO) was obtained in our laboratory by an original process based on the co-transfection of dicistronic expression vectors followed by a dual-marker amplification strategy. This led to a secretion level which is 2-3 fold higher than the values already reported for the expression of this glycoprotein hormone. Laboratorial and pilot scale of CHO cell culture were set up, in flasks and in hollow-fibre bioreactor, with a production of at least 0.5 and 1 mg hTSH/day respectively. A 4-step purification strategy was developed which provided immunologically and biologically active purified hTSH (chemical reagent level), its purity and identity being evaluated by physico-chemical, immunological and bioassay methods, all of them developed in our laboratory for this purpose. Related researches, aiming always at the success of hTSH synthesis, characterization and application were developed: (I) A novel highly sensitive, precise and accurate qualitative and quantitative analytical HPLC technique was set up, which allowed the analysis of hTSH in conditioned CHO medium even before purification. This same system, when applied to the purified material, resulted in a precious tool for the quality control of the final product. (II) A purification strategy for rapidly obtaining recombinant human thyrotropin (r-hTSH) was designed based on size exclusion and reversed-phase high performance liquid chromatographic (HPLC) analysis, carried out on hTSH-secreting CHO cell conditioned medium. These analyses permitted the identification of the main contaminants to be eliminated. Considering that hTSH is highly hydrophobic and elutes only with the addition of organic solvents, hydrophobic interaction chromatography was adopted as the first purification step; this resulted in the elimination of, among others, the major contaminant. A second purification step based on size exclusion chromatography, was then utilized being effective in the elimination of other previously identified contaminating proteins. Useful purity, as high as 99% at the chemical reagent level, and recoveries (37%) were obtained by adopting this two-step strategy, which also provided adequate material for physicochemical, immunological and biological characterization. This included matrix-assisted laser desorption ionization time-of-flight mass spectral analysis (MALDI-TOF-MS), Western blotting analysis, *in vivo* biological assay, size exclusion HPLC (HPSEC) and reversed- phase HPLC (RP-HPLC) analysis, which confirmed the integrity and bioactivity of our r-hTSH in comparison with the only two reference preparations available at the milligram level of native (NIDDK) and recombinant (Thyrogen) hTSH. Thyrogen and r-hTSH-IPEN, when compared to pit-hTSH-NIDDK, presented more than twice as much biological activity and about 7% increased molecular mass by MALDI-TOF-MS analysis, an accurate heterodimer mass determination providing the Mr values of 29 611, 29 839 and 27 829, respectively. The increased molecular mass of the two recombinant preparations was also confirmed by SDS-PAGE and HPSEC analysis. Comparing the two recombinant preparations, minor though interesting physico-chemical and biological differences were also observed. (III) A special strain of CHO cells that is capable of producing humanized glycoprotein hormones in general (i.e. whose carbohydrate structure is more similar to the human) has been prepared and work is being developed in order to obtain useful amounts of humanized hTSH.