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Hormonal effects on the secretion and glycoform profile of corticosteroid-binding globulin

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Abstract

Corticosteroid-binding globulin (CBG) is a plasma glycoprotein that is primarily synthesized in the liver and binds cortisol and progesterone with high affinity. In this study, a CBG secreting hepatocellular carcinoma derived cell line (HepG2) was used to investigate the hormonal regulation of hepatic CBG synthesis. HepG2 cells were grown for 72 h in 30, 300 and 3000 nM concentrations of estradiol (E_2), testosterone (T), insulin, thyroxin (T_4) and dexamethasone (DMZ) and the secreted CBG quantified by a novel enzyme-linked immunosorbent assay (ELISA). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was carried out to determine the effects of these hormones on the relative distribution of CBG glycoforms.

Insulin, T_4 and high concentrations of E_2 decreased the secretion of CBG by HepG2 cells ($p < 0.05$). Ethanol, the solvent used for E_2, T and DMZ, also significantly attenuated CBG secretion. 2D-PAGE resolved 13–14 glycoforms of CBG produced by HepG2 cells. Insulin caused a reduction in the synthesis of more acidic, while T_4 and DMZ decreased the production of more basic CBG glycoforms. Stimulation with E_2 resulted in the synthesis of additional isoforms of increased acidity, which may represent a type of CBG only seen during pregnancy *in vivo*. Possible physiological implications of these findings are discussed.

Keywords: Corticosteroid-binding globulin (CBG); HepG2; Hormonal regulation; Two-dimensional polyacrylamide gel-electrophoresis

1. Introduction

Corticosteroid-binding globulin (CBG, transcortin) is a 50–60 kDa plasma transport glycoprotein that binds cortisol and progesterone with high affinity [1]. About 90% of circulating cortisol is bound to CBG, where it occupies more than half of the available binding sites [2], with the remaining cortisol either bound to albumin or free [3].

Historically, two models have been employed to explain the functional significance of plasma transport proteins such as CBG. The free hormone hypothesis states that steroids bound to plasma binding proteins are unavailable to tissues, and that only the unbound fraction can cross cell membranes and thus be biologically active [3]. According to this hypothesis, the primary role of CBG would be to regulate the bioavailability and metabolic clearance of glucocorticoids, by transporting them in the blood and thus protecting them from absorption into cells and degradation by chemicals and enzymes.

Over the last 30 years, however, a large volume of work has been published, which suggests that plasma binding proteins such as CBG play a much more active role in steroid-tissue interactions. These findings resulted in the formulation of the bound hormone hypothesis, according to which hormones can also exert certain biological functions while attached to carrier proteins like CBG. In the case of CBG, the most important, development leading to this realization was the discovery of specific, high-affinity CBG receptors on the plasma membranes of various types of cells [4,5]. The bind-

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ing of CBG to these receptors can trigger various events such as internalization of the CBG-steroid complex [6], the release of bound steroids for localised delivery [7] or the induction of second messenger systems [8].

Intact CBG consists of two broad electrophoretic variants [9] and up to 13 isoforms that can be resolved by two-dimensional polyacrylamide gel-electrophoresis (2D-PAGE) [10,11]. The appearance of these different isoforms is most likely due to differences in glycosylation. CBG contains six N-linked consensus sites for glycosylation [12], five of which are usually occupied with biantennary or triantennary oligosaccharides [13]. Altered glycosylation patterns have been observed during fetal development [14], pregnancy [15] and in certain diseases [16]. Glycosylation may increase CBG half-life [17] and play an important role in membrane interactions [18] as well the cellular secretion of CBG [19].

The primary site of CBG biosynthesis is the liver [20], where CBG secretion is tightly regulated by various hormones [1]. Although numerous studies have examined hormonal effects on CBG biosynthesis *in vitro* and *in vivo*, the results of these studies have not always been consistent.

The aim of the present study was to further investigate the hormonal regulation of hepatic CBG biosynthesis using a hepatocellular carcinoma derived cell line (HepG2) and to assess the effects of these hormones on the glycosylation patterns of CBG.

2. Materials and methods

2.1. Materials

RPMI 1640 and cell culture reagents, thyroxin (3,3′,5,5′-tetraiodo-L-thyronine), testosterone (17β-hydroxy-3-oxo-4-androstene), dexamethasone (9α-fluoro-16α -methylprednisolone), bovine insulin, streptomycin sulphate, bovine serum albumin (BSA), goat anti-rabbit IgG antibody conjugated to horseradish peroxidase, donkey anti-sheep IgG antibody conjugated to alkaline phosphatase, pH 3–10 carrier ampholytes and general laboratory chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Estradiol was from BDH biochemicals Ltd. (Poole, UK). Fetal bovine serum (FBS) and Amphotericin B was obtained from GIBCO laboratories (Grand Island, NY, USA). Culture flasks were from Nunc (Roshilde, Denmark). Kanamycin sulphate was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Immobilon^{TM}-P^{SQ} polyvinylidene fluoride (PVDF) membranes and Amicon® Ultra centrifugal filter devices were from Millipore (Sydney, Australia). BCIP (5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt), NBT (*p*-nitro blue tetrazolium), Bradford protein assay reagent, pH 3–10 immobilized pH gradient (IPG) strips and 30% acrylamide/bis solution were from Bio-Rad (Sydney, Australia). The HepG2 cell line (HB 8065: American Type Culture Collection, Rockville, MD, USA) was kindly provided by the Garvan Institute of Medical Research (Sydney, Australia), where it had been tested for contamination with mycoplasma. Purified human CBG and polyclonal rabbit anti-human CBG antibody was purchased from Affiland (Ans-Liege, Belgium). Polyclonal sheep anti-human CBG antibody was from Cortex Biochem (San Leandro, CA, USA).

2.2. Cell culture

The HepG2 cell line (one clone) was grown in T75 EasYFlasks^{TM} at 37 °C, 5% CO_2 and 95% humidity in RPMI 1640 supplemented with 10% FBS and 20 mmol/L L-glutamine with media changes every 48 h. Adherent, confluent cells were harvested by washing twice with phosphate-buffered saline (PBS) containing 0.25% trypsin–ethylenediamine tetraacetic acid (EDTA) and frozen to provide cell stocks with identical passage history. For stimulation experiments, frozen cell stocks were thawed and plated at approximately equal numbers of approx. 10^6 cells per flask and grown to about 70% confluence. The cells were then washed twice with PBS and incubated for 24 h in FBS-free medium supplemented with amphotericin B (1 μg/mL), kanamycin sulphate (100 μg/mL) and streptomycin sulphate (100 μg/mL). Medium was aspirated followed by a further 72 h in FBS free medium in the presence or absence of hormones. Duplicate flasks were used for each hormone concentration and their respective controls.

2.3. Hormonal stimulation

Stock solutions of estradiol (E_2), testosterone (T) and dexamethasone (DMZ) were prepared in ethanol (ca. 3 mg/mL), while thyroxin (T_4) and insulin were dissolved directly in FBS free medium. Hormones were added to the cell cultures to final concentrations of 30, 300 and 3000 nmol/L. Controls were conducted with support medium only and with medium supplemented with ethanol to final concentrations of 80, 800 and 8000 nmol/L, the same amounts of ethanol that had been added to the cultures stimulated with 30, 300 and 3000 nmol/L, respectively of DMZ, E_2 and T. This was done to allow comparison of each cell culture stimulated with ethanol dissolved hormones to a control containing the exact same concentration of ethanol.

After 72 h the medium was removed and centrifuged at 2500 g for 5 min at 4 °C and the supernatant stored at −20 °C until analysis. To correct for variations in cell numbers/culture the adherent cells were washed twice with PBS and harvested using 0.25% trypsin–EDTA. The cells were then lysed for 2 h at 60 °C in 0.1 M NaOH and stored at −20 °C awaiting analysis of total cellular protein.

2.4. CBG ELISA

CBG in media samples was analysed by enzyme-linked immunosorbent assay (ELISA). Briefly 50 μL of polyclonal rabbit anti-human CBG antibody diluted 1:150 in carbonate/bicarbonate buffer (50 mM $NaCO_2$–$NaHCO_2$, pH 9.6)