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Estrogen dependent expression of sex hormone binding globulin in PC 12 cells

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ABSTRACT

Rat pheochromocytoma PC 12 cells are known to develop features of dopaminergic neurons upon treatment with nerve growth factor. They express in part estrogen receptors α and β, and G-protein coupled receptor 30. Estrogens promote development of these cells and exert neuroprotective effects. Here we treated differentiated PC 12 cells with physiological concentrations of 17-β-estradiol. We observed with immunocytochemistry cytoplasmic staining for SHBG in a portion of these cells Double immunostaining for estrogen receptor-β revealed that some PC 12 cells contained both antigens. Numbers of estrogen receptor-β positive cells were significantly higher after estradiol treatment; an effect that was not altered by pretreatment of cultures with tamoxifen. With reverse transcriptase polymerase chain reaction we observed sex hormone binding globulin encoding transcripts indicating intrinsic expression of the steroid binding globulin. We conclude that estrogen treatment induces SHBG expression in differentiated PC12.

1. Introduction

Sex hormone binding globulin (SHBG) is a 94-kDa homodimeric glycoprotein with specific binding affinities for androgens and estrogens [1]. Intrinsic expression of SHBG was found also in organs other than the liver including pituitary, adrenals, ovaries, prostate and cardiomyocytes [2–6]. SHBG seems to play a role in various pathologies including breast cancer, also effects on aging, osteoporosis, obesity, metabolic syndrome and reproductive organ pathologies. Several studies suggested the functional importance of SHBG in the central nervous system, e.g. in the pathologies of Alzheimer's disease [7–11].

In previous studies we demonstrated that SHBG is expressed in various brain regions in rat and in humans [12–14]. SHBG expression rates in brain and cerebrospinal fluid (CSF) levels of SHBG are malleable to changes of endocrine state like pregnancy, parturition, nursing and stress [12]. We observed in magnocellular hypothalamic neurons the coexpression of SHBG with peptide hormones of the neurohypophyseal system. In oxytocinergic neurons SHBG was found in secretory vesicles along with oxytocin, indicating that SHBG is subject to axoplasmic transport and terminal release in a neurohormone-like fashion [13]. SHBG has been demonstrated to be taken up by various brain cells including neurons of the PVN [30]. This internalization seemed to be associated with the presence of estradiol receptor-β [30]. Therefore, it appears that SHBG is actively involved in at least some steroid effects in brain and may serve to deliver steroids to various intracellular sites. Although SHBG expression is estrogen dependent in liver and perhaps also in brain, most of the SHBG expressing neurons proved to be devoid of nuclear ER immunoreactivity. Behavioral experiments indicated that SHBG may be an actor in the non-genomic rapid response to estrogen treatment (for review see [14]).

We wanted to study the underlying cellular and molecular events on the single cell level in a cell culture system. One of the best characterized neuron like cell lines is the PC 12 line which is derived from a rat pheochromocytoma. PC 12 cells have been shown to develop features of dopaminergic neurons upon treatment with nerve growth factor NGF [15–17], which causes the cells to differentiate into neuron-like cells. Neurite formation and differentiation can be enhanced in these cells by estrogens [18]. Estrogens also seem to exert neuroprotective effects [19,20]. Some PC 12 cells express nuclear receptors for estrogens ER-α and ER-β [21]. In this study we wanted to examine SHBG expression in NGF-treated PC 12 cells with light microscopical immunocytochemistry and RT-PCR. Special attention was paid to the possible colocalization of SHBG with ER-β. We further addressed the questions of whether treatment with estradiol or its antagonist tamoxifen would affect SHBG expression in PC 12 cells.

Abbreviations: PC 12, rat pheochromocytoma cells; NGF, nerve growth factor; ER, estrogen receptor; GPR30, G-protein coupled receptor 30; SHBG, sex hormone binding globulin; E2, 17-β-estradiol; CSF, cerebrospinal fluid; RT-PCR, reverse transcriptase polymerase chain reaction; DMSO, dimethylsulfoxid; CBG, corticosteroid binding globulin.

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2. Materials and methods

17-β-estradiol and tamoxifen were dissolved in Dimethylsulfoxide (DMSO), to a concentration of 10^{-4} M (estradiol) and 10^{-5} M (tamoxifen). Steroids were added to cell culture medium (RPMI 1640 Biochrom GmbH) containing 10% fetal bovine serum (FBS, Sigma-Aldrich) and applied 5 mM L-glutamine to a final concentration of 10^{-6} M. The final concentration of NGF in culture medium was 50 ng/ml (all reagents obtained from Sigma–Aldrich, Munich, Germany).

PC12 cells were a gift from Prof. Dr. Grune, Friedrich-Schiller-University Jena, Institute of Nutrition, Dept. of Nutritional Toxicology, Germany. Cells were grown in36 mm polystyrene culture dishes (Falcon®) under 5% CO_2 in air at 37 °C. The medium was changed daily. For passage cells were trypsinized at 80% confluence. NGF treatment was started after two days *in vitro* and continued for six more days. The medium contained in addition either estradiol, Tamoxifen or estradiol and tamoxifen combined. Controls were carried out with culture medium that contained only DMSO in the respective concentration without steroid.

For immunocytochemistry cells were fixed with 4% paraformaldehyde (PFA, Roth, Germany) in PBS (0.1 M sodium phosphate buffer pH 7.2, containing 0.9 M NaCl) for 15 min at RT. Thereafter cultures were washed 3× with PBS containing 0.1% TRITON® X-100 (PBS-Triton, Merck Millipore, Germany) for 5 min each. Then cells were incubated with either rabbit antibody to neurofilament diluted 1:1000 in PBS (Chemicon, Temecula, USA) or with rabbit anti-SHBG or rabbit anti-ER beta (polyclonal antibody, diluted 1:500 in PBS, Santa Cruz Biotechnology) overnight at 4 °C. After washing in PBS-Triton, incubations were performed with goat anti rabbit IgG followed by rabbit peroxidase-anti-peroxidase (PAP) complex. Both detection antibodies were diluted in PBS-Triton 1:200 and applied for 1 h at RT each. Immunopreciptiates were visualized with diaminobencidine (DAB) and H_2O_2 (Sigma FAST Kit). All reagents obtained from Sigma–Aldrich, Munich, Germany.

For double immunostaining cultures were incubated for 10 min at RT in 0.1 M glycine buffer pH 3.5 to remove the first antibody complexes. After washing in PBS-Triton cultures were incubated with rabbit anti ER-β diluted in PBS-Triton 1:500 (Santa Cruz Biotechnology) overnight at 4 °C. After washing in PBS-Triton cultures were stained with either CY3- or Alexa 488 labeled anti rabbit IgG at a dilution of 1:100 in PBS-Triton for 1 h at RT. After washing in PBS cultures were mounted with an aqueous mounting medium (10% Mowiol, plus 10 μg/ml DAPI nuclear stain, Roth, Germany) and examined with epifluorescence or transmission illumination microscopy. In some experiments ER-β was stained with the PAP method, followed by SHBG immunofluorescence. A Zeiss Axiovert photo microscope was used for examination, counting and photography of stained cell cultures.

RNA of PC-12 cells was isolated by TRIzol® extraction. After dissolving the cell layer in 3 ml TRIzol®, samples were collected in reaction tubes and homogenized by sonication. RNA from rat liver tissue was used as SHBG-positive control. 200 mg of fresh liver that had been briefly perfused with sterile PBS was placed in a reaction tube with 3 ml TRIzol® and homogenized mechanically (Ultraturrax T3) followed by sonication. Homogenized samples were incubated for 5 min at RT. 0,6 ml chloroform was added and the tubes were shaken by hand for 15 s. After an incubation of 3 min at RT samples were centrifuged at 12,000*g* for 15 min at 4 °C. The aqueous phase, containing the isolated RNA, was placed into a new tube. For RNA precipitation samples were mixed with 1.5 ml of 100% isopropanol, incubated for 10 min at RT and then again centrifuged for 10 min at 12,000*g* at 4 °C. Supernatant was removed from the tube. The pellet was resuspended by briefly vortexing in 3 ml of 75% ethanol. After centrifugation (7500*g*, 5 min, 4 °C) the supernatant was discarded. The air-dried pellet was resuspended in 50 μl of RNase-free water and incubated in a heat block at 55 °C for 15 min.

Reverse transcription and PCR were conducted with QIAGEN OneStep RT-PCR Kit. The used primer sets for SHBG and ER as well as for β-actin, are available from Biomol GmbH, Germany. The following primer sequences for SHBG (rat) were used: forward primer: 5′-CCA AAC GGT GGT TCT GTC TT-3′ and reverse primer: 5′-TAA AGC CCC AAG GGA GAG AT-3′ with a product size of 208 bp. The primers for the housekeeping gene β-actin (rat) were: forward primer: 5′-CAC ACT GTG CCC ATC TAT GA-3′ and reverse primer: 5′-CCG ATA GTG ATG ACC TGA CC-3′ with a product size of 272 bp.

The cycling conditions (Biometra T3 Thermocycler) included 50 °C for 30 min for the reverse transcription and 95 °C for 15 min as the initial PCR activation step, followed by a 3-step cycling of 94 °C for 30 s, 53 °C for 45 s and 72 °C for 1 min (35 cycles), and a final extension step of 72 °C for 10 min. The final PCR product was separated in an agarose-gel (1.2%). The amplification product was visualized with ethidium bromide under ultraviolet light. Standard DNA size markers are used to estimate the size of the PCR product (Low range DNA ladder, Jena Bioscience, Germany).

3. Results

After 6 days of NGF treatment PC 12 cells developed heterogeneous morphologies including bipolar or multipolar neuron like cells, some of them extending relatively long processes. Most of these cells exhibited strong neurofilament immunoreactivity in perikarya and processes (Fig. 1A) indicating that they were becoming neuron-like. Immunofluorescence for SHBG was found in the perinuclear cytoplasm and also in nuclei in about 9.8% of the PC 12 cells, including the ones that appeared to be neurofilament negative (Fig. 1B). 3.2% of PC 12 cells showed nuclear immunoreactivity for ER-β (Fig. 2). Double immunostaining revealed some colocalization of SHBG and ER-β (Fig. 3). Most of the SHBG-positive cells were ER-β negative and vice versa. After six days of estrogen treatment numbers of SHBG immunostained PC 12 cells were increased to 72%. Overall staining intensity of SHBG immunoreactivity also appeared to be increased (Fig. 4) as compared with the DMSO treated controls. Numbers of ER-β immunoreactive cells were also increased after E2 treatment to 35%. The proportions of cells that contained both immunoreactivies were slightly increased after estrogen treatment with SHBG and ERβ mostly exclusive. PC 12 cells showed similar immunoreactivity patterns when treated with estradiol regardless of whether cells were pre-treated with tamoxifen (Fig. 5A and B).

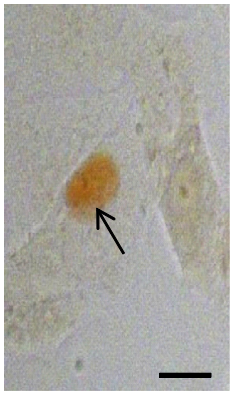

Fig. 1. Nuclear immunostaining for ER-β is found in a small fraction of untreated PC12 cells (arrow) while most of the cells are devoid of such staining. Scalebar = 5 μm.