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The Close Proximity of Sex Hormone Binding Globulin and Estradiol Receptor-beta (ERβ) in PC12 Cells Differentiated with Nerve Growth Factor and Treated with Estradiol

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Abstract

We have demonstrated the production and distribution of Sex Hormone Binding Globulin (SHBG) in the brain. We have also seen that SHBG is internalized into specific brain cells including neurons and that this internalization appears to be linked to the presence of Estradiol Receptor Beta (ERβ). Recently, we demonstrated that differentiating PC12 pheochromocytoma cells into neuron-like cells with Nerve Growth Factor (NGF) and treating them with estradiol elicited expression of both SHBG and ERβ in these cells. This manuscript further confirms that work using two different antibodies for SHBG and ERβ. The purpose of these experiments is to determine whether this co-localization of SHBG and ERβ is of a very close proximity to eventually interact. Next we use confocal microscopy to show that, not only are these two steroid binding proteins found in the same cells, they are found very close to each other near the cell's plasma membrane. Finally, we use the Duolink® technology to demonstrate that SHBG and ERβ are in extremely close proximity (<30 nm) of each other in these cells. Further elucidation of the functional significance of SHBG being in close proximity with ERβ awaits other studies.

Introduction

Sex Hormone Binding Globulin (SHBG) is made in great quantities in the liver [1-3], but is also made in the brain [4-6], [7-9]. We have demonstrated SHBG in other non-liver sites including the male reproductive tissues [10], the nose [11,12], and the heart [13]. In the old model, steroid binding globulins were only useful to carry their associated steroids around in the blood only to drop them off near their target cells to let them passively diffuse across the target cell's plasma membrane to act on cytoplasmic receptors such as Estradiol Receptor Beta (ERβ). This old dogma dictated that steroid binding globulins played no role in the diffusion and targeting of the steroid to its receptor. However, today a much more complex view of steroid-binding globulin action is realized. We have expressed on numerous occasions [14-18], that SHBG and other steroid binding globulins play a much more active role in steroid actions.

As part of this more active role, we have suggested there is an SHBG receptor [15,17,19,18]. We also postulated a critical role for ERβ in the internalization of SHBG [20]. The existence of an SHBG receptor associated with the cell's plasma membrane has been suggested by other laboratories [21-28]. Previously, we found that intracerebrally injected fluor-labeled SHBG was internalized into specific neuronal cells [29]. In that same manuscript, we demonstrated that SHBG was only internalized by mouse hippocampal cells stably transfected with cDNA for ERβ. SHBG was not internalized by wild-type cells or cells stably transfected with cDNA for ERα [29]. This suggested that there is a special relationship between SHBG and ERβ. This relationship

could either be that the presence of ERβ caused the production of something that allowed SHBG internalization or that ERβ was part of a greater complex with SHBG that allowed its internalization. More recently we suggested that SHBG, like other steroid binding globulins [17,20], is very important in the internalization of the steroid itself.

This relationship of SHBG and ERβ was further examined by Gebhart and Jirikowski [30], who showed that PC12 cells differentiated with nerve growth factor take on neuron-like characteristics, and when treated with estradiol demonstrated both SHBG and ERβ immunoreactivity. In this manuscript, we attempt to determine if SHBG and ERβ are localized close enough PC12 cells to actually physically interact with each other.

Materials and Methods

Cell Culture

Pheochromocytoma (PC12) cells provided by Dr. H. Macarthur (Saint Louis University, St. Louis, MO) were grown in high glucose (4500 mg/L) DMEM supplemented with 2 mM glutamine, 1 mM pyruvate, fetal bovine serum (5%) and heat-inactivated horse serum (10%) in a humidified 5% CO_2 atmosphere at 37°C. Cells were passaged at 1-week intervals by dissociation with cell culture media and manual trituration. Media was changed every 2-3 days. For experimentation PC12 cells were plated at a density of 1.6 x 10^4 cells/chamber in poly-l-lysine coated 8-chambered microscope slides (Nunc cat# 154941). All cells were treated with NGF (final concentration of 200 ng/ml) to differentiate them into neuron-like cells. Estradiol (E2) treatment wells received E2 at a final concentration of 10^{-6} M added to the growth media as described in Gebhart and Jirikowski [30]. Each chamber contained 400 µl of DMEM. After 5 days of incubation in a CO_2 controlled incubator at 37°C with NGF alone or with E2 the cells were treated as required for each particular study.

Immunocytochemistry

After 5 days of incubation at 37°C with NGF and E2 as described above, the media was aspirated off and cells were fixed with 10% paraformaldehyde for 10 minutes at Room Temperature (RT). After 2 PBS washes, cells were exposed to both an ERβ antibody (Santa Cruz ERβ mouse monoclonal antibody SC373853 at a 1:200 dilutions in PBS) and an SHBG antibody (Santa Cruz SHBG rabbit monoclonal antibody SC32891 at a 1:250 dilutions in PBS) over night at 4°C. The solution was then aspirated and the cells washed with PBS 3 times for 3 minutes each. Negative controls were run where equal volumes of vehicle were added to chambers instead of primary antibodies. For light microscopy, secondary antibodies with differently colored fluorochromes were added to the chambers. Alexa Fluor 488 Goat anti - mouse IgG (Molecular probes, cat#A11029) was used to identify the ERβ primary and Alexa Fluor 594 goat Anti - rabbit IgG (Molecular probes, cat# A1102) was used to identify the SHBG primary (at 1:200 dilutions for both) were added to the chambers for 1 hour at RT under minimal light exposure. After 1 hour, the cells were washed with PBS - Tween for 3 washes of 3 minutes each. DAPI was either added directly to the cells at this point or chambers were mounted with DAPI-containing mounting media (Vector Labs, Vectastain, aqueous mounting media with DAPI, cat#H1200). Slides were then examined using an EVOS inverted light microscope (EVOS FL AUTO).

Laser Confocal Microscopy

PC12 cells were treated as indicated above in 8-chambered microscope slides. Also, cells were affixed to the slides with paraformaldehyde as indicated for regular light microscopy above. Once treatments were finished and cells affixed, the chambers of the 8-chambered slide were removed and slides were immediately mounted with DAPI-containing aqueous mounting media (Vector Labs, Vectastain, aqueous mounting media with DAPI, cat#H1200) affixing the coverslip to the slide at the corners. Appropriate cells were identified using the light microscope aspect of the confocal microscope before analyzing single cells with the laser confocal microscope (Leica TCS SM8). Once an appropriate cell or portion of a cell was identified, a series of confocal images were taken sequentially, essentially giving a 3-dimensional view of the cell or a section of the cell. This series of views (the z-stack) was then rotated computationally using the free ImageJ® program from NIH, to give a "side-on" or orthogonal view. This allowed us to distinguish whether double-labeling was actually occurring as opposed to simply appearing to be in close proximity with one fluorochrome over top of the other.

Duolink® Experiments

For the Duolink® experiments, PC12 cells were differentiated and treated as described in the ICC studies, and the same primary antibodies were used for both SHBG and ERβ at the same dilutions. After treating PC12 cells daily with NGF alone or NGF plus E2 for 5 days as indicated above, the media was aspirated and cells were fixed with 10% paraformaldehyde added to each chamber for 10 minutes at RT. The instructions for the Duolink® kit (Sigma catalog #DUO82040) were then followed. Briefly, the Duolink® proximity ligation assay uses secondary antibodies (anti-mouse and anti-rabbit PLA© probes at 1:5 dilution) that are conjugated to cDNA oligomers. Both of the two separate oligomers are required to be in close proximity (<30 nm) for other oligomers, provided in the kit, to hybridize to form a complex. Then a ligase enzyme was used to form a closed, circularized DNA template, which is required for rolling circular amplification. This was incubated with the cells (1:40 dilution) for 60 minutes at 37°C. Next, DNA polymerase was added (1:5 dilution). This DNA probe serves as a primer for the rolling circular amplification and generates a single-stranded concatemeric sequence of up to 1000 copies of the template (hence the amplification of signal). This amplicon remains tethered to the secondary antibody template. Cells were incubated with polymerase for 30 minutes at 37°C. Finally, labeled oligomers, or detection probes, were hybridized to the amplicon

for 100 minutes at 37˚C. If the primary antibody-labeled molecules are within 30 nm of each other, then a red dot is visible.

Results

Confocal Analysis of the Co-localization of SHBG and ERβ

First, we confirmed the Gebhart and Jirikowski [30] finding that there is immunoreactivity for both SHBG and ERβ in PC12 cells. Then we began analyzing neuronally differentiated PC12 cells for these elements using laser confocal microscopy. Figure 1 is a confocal micrograph of NGF-differentiated PC12 cells treated with E2. It is apparent that both SHBG and ERβ are found in processes, which further suggests that they are localized either in the cytoplasm or at the cell membrane and not associated with the cell's nucleus. Careful analysis of the XY and YZ orthogonal views of this confocal image, shows that at least some SHBG and ERβ immunostaining is found on the edge of the cell, likely associated with or at least near the plasma membrane. The SHBG appears as red fluorescence whereas the ERβ immunofluorescence is green. Because these two were aligned along the line of sight in the normal view, they instead appear as yellow. It is only by using the confocal microscope with its ability to show "side-on" orthogonal views that it becomes apparent that these two are very near each other with one "on top" of the other.

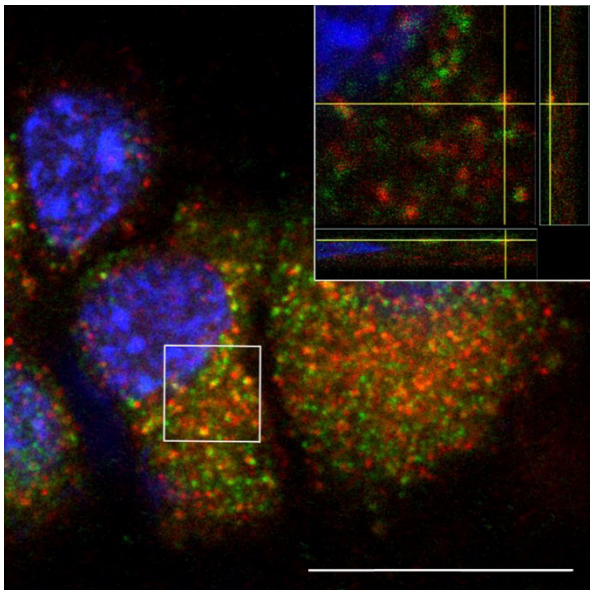
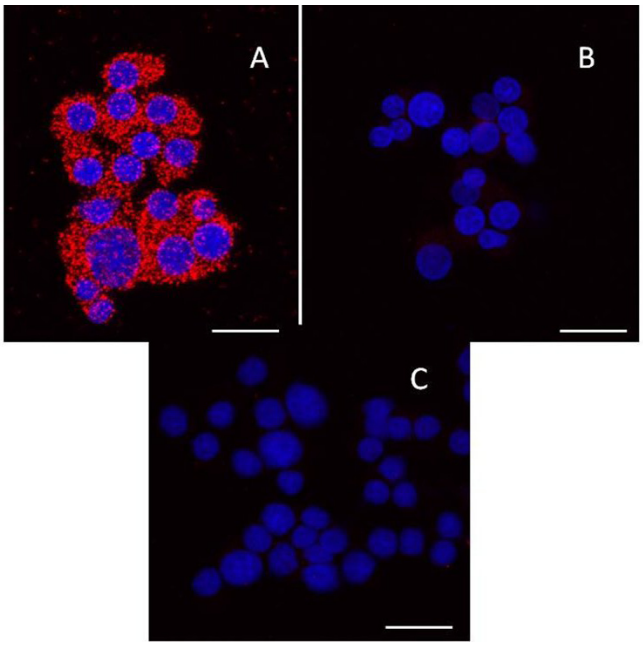


Figure 1: A confocal micrograph of NGF-differentiated PC-12 cells showing SHBG (red); and ERβ (green) co-localization. The inset in the upper right is a magnification of the area indicated with a white square. Orthogonal views of this inset area are shown to the right (YZ axis) and below (XZ axis). The yellow crosslines in the orthogonal views are crossing directly over closely co-localized SHBG and ERβ. It appears that both of these lie very near the surface of the cell. DAPI nucleus marker is in blue. Scale bar = 5 µm.

Duolink® Analysis of the Proximity of SHBG and ERβ

In Figure 2A there is a high density of red dots associated with NGF-differentiated PC12 cells also treated with E2. These red dots were only found around and associated with cells as identified by DAPI-stained cell nuclei indicating they were not artefactual. Because of the nature of the Duolink® technique, these red dots indicate that the two elements probed by primary antibodies for SHBG and ERβ, are in very close proximity, i.e. less than 30 nm apart. Figure 2B and Figure 2C show that without both antibodies present (negative controls) there were no red dots present. The advantage of viewing these cells with confocal microscopy allows the viewer to see "through" the cell and thus have a better idea of where these red dots are appearing relative to the edges of the cells and where cells are contacting each other (because they are in clusters). It was very apparent to the viewers that these red dots were away from the nuclei and were generally found at what appeared to be the outside of the cell. This would suggest that these red dots were near or in the cell's plasma membrane. We analyzed the number of molecules demonstrating proximity of SHBG and ERβ per cell between those chambers treated with NGF alone versus those treated with NGF and E2 (data not shown). From this analysis, it appeared that there were more close proximity interactions in NGF-differentiated cells that were also treated with E2 than those that were not. (Figure 3) shows a diagram of interactions of SHBG and ERβ either in the cytoplasm or near the plasma membrane. From our current findings an interaction near the cell's plasma membrane appears better supported.



Figures 2(A-C): Laser confocal images of three treatments using the Duolink® technique. In the presence of both the SHBG (see Methods for details) and ERβ antibodies (A), with the SHBG antibody only (B) or the ERβ antibody only (C). It is clear that there are numerous distinct red dots with both antibodies present (A), indicating that the SHBG and ERβ molecules are often within 30 nm of each other. Scale bars = 10 µm.

Discussion

These studies clearly indicate that our hypothesis that SHBG and ERβ are in very close proximity in PC12 cells is correct. Gebhart and Jirikowski [30] first demonstrated that SHBG and ERβ were co-localized in NGF-differentiated PC12 cells treated with E2. Our current paper corroborates those findings using different antibodies from those used by Gebhart and Jirikowski [30]. Further, the confocal images from this study more specifically determine that SHBG and ERβ are closely associated and that often this association takes place in cellular processes and near the plasma membrane. Using the Duolink® technology, which is specifically designed to detect when two molecules are within 30 nm of each other, we found that SHBG and ERβ often are in very close proximity with each other. (Figure 3) shows a model wherein SHBG and ERβ in close proximity with each other, and suggests that this could be happening either near the plasma membrane, or within the cytoplasm. Careful analysis of both the confocal microscopy and Duolink® results suggests that their interaction is at the level of the plasma membrane.

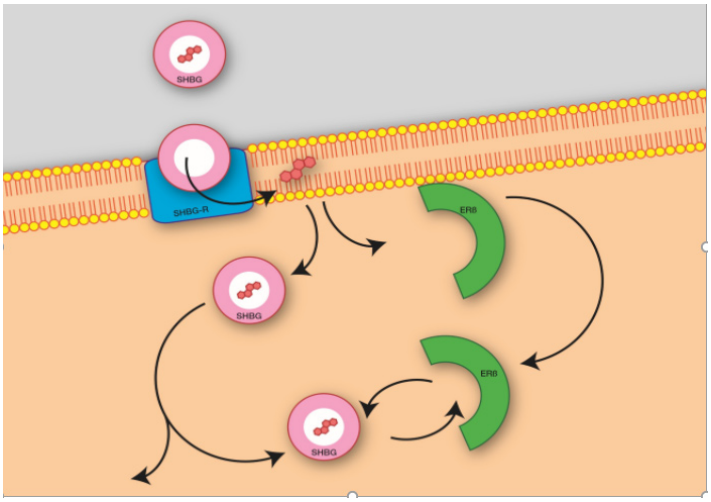


Figure 3: This is a diagram of our model for the interaction of SHBG and ERβ, which is important for E2 uptake into the cell and for its effects on the cell. SHBG (pink) carries estradiol (the red four-ring structure) in the blood and extracellular fluid. SHBG is then bound by the SHBG receptor (light blue) in the plasma membrane. Estradiol is released from SHBG directly into the lipid bilayer as with retinol in the Kawaguchi model [40]. From the lipid bilayer, estradiol must be taken up by a protein. In this model this can be either intracellular SHBG or ERβ (green) located on the inside of the plasma membrane. It is quite likely that ERβ is actually part of a protein complex that includes the SHBG receptor and thus is much closer to the SHBG receptor than is depicted here. Finally, and alternatively, it is also possible that intracellularly SHBG delivers E2 to the cytoplasmic ERβ.

Several laboratories have suggested that estradiol receptors are found near, and in fact are specifically trafficked to, the cell's plasma membrane [31-36]. There is a discussion as to whether the classic cytoplasmic estradiol receptors (ERα and β) gain exposure to the outside of the cell's membrane, but there is some consensus that they are at least found near the inside of the plasma membrane. It has also been suggested that ERs are found associated with caveolae [34,37,38]. Kawaguchi et al. [39,40] proposed a model wherein the steroid binding globulin Retinol Binding Protein (RBP) is bound by a membrane protein called STRA6. Then from the bound RBP, the steroid is released into the bilipid plasma membrane, from which other proteins must bind it in order for it to enter the cell. We suggest that a similar model is appropriate for SHBG and ERβ. As suggested above, there is a membrane-associated receptor for SHBG. We have some tentative evidence for this SHBG receptor in brain cells [15,41]. More recently we postulated that SHBG binding to a membrane-associated protein was important for the internalization of estradiol and further suggested that this was part of a larger protein complex, which included ERβ [17]. Our current finding that SHBG and ERβ are often found in very close proximity, and apparently frequently near the plasma membrane, supports this model.

The PC12 cells used in this study are an immortalized cell line derived from a pheochromocytoma tumor of the rat adrenal gland [42]. Perhaps for this reason, when they are treated for several days, as we do in this experiment, with NGF they differentiate into neuron-like cells with processes [43]. When PC12 cells are treated with NGF the cell bodies flatten, become elongated, and develop neurite processes [43]. Additionally, PC12 cells develop neuronal characteristics such as regulated Ca^{2+}-dependent vesicular neurotransmitter release [42]. Here we demonstrated that both SHBG and ERβ are often co-localized in these processes, apparently near the plasma membrane. While the phenomenon of SHBG being in close proximity to ERβ is of significance for a wide range of steroid-sensitive cells, it is of particular interest to us if these two interact in neurons. We have demonstrated the production of SHBG in neurons [5,15,44], as well as uptake of SHBG into neurons and into mouse hippocampal HT22 cells [29], suggesting that SHBG has a clear function in steroid actions in the brain. That internalization of SHBG into HT22 cells only occurred if these cells were stably transfected with cDNA for ERβ [29], suggested that there is a special and particular relationship between SHBG and ERβ that is critical for the uptake of steroids into neurons, as we have previously postulated [17].

Our previous work with video imaging of fluorochrome-labeled E2 demonstrated that applying SHBG antibody to E2-sensitive cells blocked or delayed uptake of E2 [17]. Therefore, we postulated that SHBG and possibly an SHBG receptor were necessary for the uptake of E2, at least in some cells. We postulated that SHBG and its putative membrane-associated receptor are part of a larger protein complex responsible for E2's uptake. We further suggest that ERβ is part of this complex. We postulate this interaction occurs along the lines of the model of Kawaguchi et

al. [40], for retinol and RBP, wherein RBP binds to STRA6, a membrane-spanning receptor, and then it releases retinol into the plasma membrane. In their model, there are intracellular proteins that are closely associated with the inner-leaf of the membrane, including a cytoplasmic RBP, which liberates retinol from the lipid membrane and transports into the cell. It is possible that ERβ is such a protein, closely associated with an SHBG receptor that is also membrane spanning with ERβ attached on the inside of the plasma membrane and critical for the internalization of E2. In any case, the current reported studies clearly indicate that these two estradiol binding proteins come into very close proximity in PC12 cells [45-51].

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