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Research Article

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Subcellular Translocations and Cross-Regulations of the Androgen Receptor and the c-AMP Response Element-Binding Protein

Masayuki Saitoh[1*], WuQiang Fan[2*], Ryoichi Takayanagi[3] Contributed Equally

[1]Department of Medicine, Saitoh Hospital, Fukuoka, Japan; [2]Division of Endocrine, Department of Medicine, Massachusetts General Hospital, Boston, MA; [3]Department of Preventive Medicine Fukuoka Sanno Hospital,Fukuoka Japan

ABSTRACT

Background: Nuclear Receptors (NRs) and G Protein Coupled Receptors (GPCRs) are two distinct but closely inter-regulated principal signal transduction pathways of eukaryotic cells. Androgen Receptor (AR) and cAMP Responsive Element-Binding Protein (CREB), are classic Transcription Factor (TF) of NRs and GPCRs pathways, respectively. While emerging knowledges suggest functional interactions between the two TFs, detailed mechanistical study is lacking.

Methods: Dynamic subcellular translocations of AR and CREB in response to activation of either and both signal pathways were studied in living cells using laser confocal microscopy. Transcriptional activities of the two TFs were assessed by activities of cognate target promoters.

Results: AR and CREB resided in cytoplasm and nucleus, respectively, both in a diffuse manner, and both were transcriptionally silent, in the absence of androgen or PKA activation. AR translocated to subnuclear foci and became transcriptionally active in the presence of Dihydrotestosterone(DHT). CREB underwent similar subnuclear foci formation and transcriptional activation in the presence of PKA stimulator forskolin (FSK). In a subset of cells where AR resided diffusely in the nucleus in the absence of DHT, FSK translocated AR to subnuclear foci and rendered AR transcriptionally active. DHT translocated both AR and CREB to around 300 subnuclear foci where the two TFs co localized. This process led to transcriptional activation of AR but not CREB. Stimulation of cells with both FSK and DHT did not alter AR-CREB co localizing foci, though the DHT mediated AR transcriptional activity was enhanced whereas FSK-induced CREB transcriptional activity was reduced.

Conclusion: Androgen-bound AR and PKA activated CREB are translocated to the identical subnuclear foci where the two TFs are subjected to mutual and differential cross-regulations such that CREB enhances DHT mediated AR transactivation whereas ligand-bound AR suppresses PKA induced CREB transactivation.

Keywords: Androgen receptor; CREB; Transcription regulation; Subcellular localization; Live cells.

Abbreviations: AR: Androgen Receptor; ARE: Androgen Responsive Element; CBP：CREB-binding protein; CFP: Cyan Fluorescent Protein; CRE: cAMP Responsive Element; CREB: cAMP Responsive Element-Binding Protein; DHT: dihydrotestosteron; ER: Estrogen Receptor; FSK: forskolin; GFP: Green Fluorescent Protein; GPCR: G Protein Coupled Receptor; GR: Glucocorticoid Receptor; HSP: Heat Shock Protein; MMTV: Mouse Mammalian Tumor Virus; NLS: Nuclear Localization Signal; NR: Nuclear Receptor; SF1:Steroidogenic Factor 1; SRC1: Steroid Receptor Coactivator 1; TF: Transcription Factor; TIF 2:Transcriptional Intermediary Factor 2.

INTRODUCTION

G-Protein-Coupled Receptors (GPCRs) are a large diverse group of transmembrane receptors [1] that respond to external signals by activating the associated G proteins, which in turn triggers the production of a variety of second messengers that regulate a wide range of cellular functions [1,2]. One particularly common target of activated G proteins is adenylyl cyclase (AC), which catalyzes synthesis of the second messenger cAMP. cAMP then activates protein kinase A (PKA), which in turn activates cAMP-Responsive element-binding protein (CREB), a nuclear protein.

transcription factor (TF) modulates transcriptions of an array of genes. nuclear receptors (NRs), on the other hand, are a family of TFs that

Correspondence to: Masayuki Saitoh, Department of Medicine, Saitoh Hospital, Fukuoka, Japan, E-mail: yugamasa31@gmail.com

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are activated by steroid hormones. Ligands for NRs, such as androgen for androgen receptor (AR), cross the plasma membrane and directly interact with NRs inside the cell. Ligand-bound NRs then directly regulate transcription of their target genes.

GPCRs and NRs thus represent two distinct categories of cellular signal transduction pathways that each regulate and control a wide variety of biological processes. Emerging evidences support the theory that the two distinct kinds of signaling are often with highly coordinated interactions and cross regulations that are of physiological and pathophysiological significances.

In this regard, it has been reported that the cAMP-PKA-CREB signaling is able to enhance androgen receptor-mediated gene transcription of the prostate-specific antigen [3] and this interaction may have relevance in prostate cancer pathogenesis. On the flip side, the androgen-AR signaling suppresses cAMP-PKA-CREB signaling in brown adipose tissue in male mice and thereby reduces β-adrenoceptor-induced UCP1 expression [4] and this mechanism may partially explain the sexual dimorphism of thermogenesis and body temperatures.

Despite apparent crosstalk and interaction between the androgen-AR and cAMP-PKA-CREB signal pathways, detailed mechanistically study at the level of transcriptional regulation is lacking.

We and others have previously reported that dynamic subcellularly imaging of the fluorescent protein-tagged NRs in living cells using laser confocal microscope is a powerful tool to study transcriptional regulation by NRs [5].

In the present study, we investigated the dynamic subcellular translocations of AR and CREB in response to activation of either and both signal pathways and report a bi-directional and differential cross-regulations between AR and CREB at the transcriptional regulation level.

MATERIALS AND METHODS

The expression plasmids for AR-GFP (pCMV-AR-GFP) and AR-CFP (pCMV-AR-CFP) chimeras were constructed by inserting the full-length AR cDNA into the NheI-SmaI sites of pEGFP-N1 and pECFP-N1 (CLONTECH Laboratories, Inc., Palo Alto, CA), respectively. The expression plasmid (pCREB-EGFP) for CREB-GFP was purchased from CLONTECH Laboratories, Inc., Palo Alto, CA. The pGL3-ARE and pGL3-CRE reporters which contain the firefly luciferase genes under the control of an androgen response element (ARE) and cAMP response element (CRE), respectively, were both gifts from Kato S (University of Tokyo). The firefly luciferase reporter vector pGL3-MMTV, which also contains response elements for AR, and the expression vectors for the human AR full length cDNA (pCMX-AR)7 were prepared as previously described8.Kidney-derived cell lines, COS7 were obtained from American Type Culture Collection (Manassas, VA). Serum free medium, GIT, was purchased from Wako Tokyo Japan for dual luciferase promoter reporter assay, COS7 cells were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% FBS, 2 mM L-glutamine and 100 U/ml of penicillin-streptomycin. Three days before experiment, medium was changed to GIT.

The cells, cultured in 6-well plates (3×10^5 cells per well), were transfected with 1 μg/well of pGL3-ARE or pGL3-MMTV or pGL3-CRE as the reporter, 2 ng/well of pRL-CMV (a Renilla luciferase vector, Promega Corp., Madison, WI) as the internal control, and 0.1 to 0.2 μg/well of the expression vector for the AR and/or the CREB, using SuperFect reagent (QIAGEN, Hilden, Germany). For co-expression studies, the total amount of vector added to each well was equalized by the addition of empty vector. Starting 3 h after transfection, the cells were incubated for 48 h in GIT in the presence or absence of 10^{-8} M of di hydro testosterone (DHT) and/or 10^{-6} M of forskolin (FSK, a direct activator of adenylyl cyclase). The cells were then solubilized with lysis buffer (Promega Corp.) and the activities of the reporter gene were determined by the Dual-Luciferase Reporter Assay System (Promega Corp.). One-way analysis of variance followed by Scheffe´'s test was used for multi-group comparisons.

Fluorescence microscopy and three-dimensional image analysis were as described previously [6]. In brief, 3×10^5 cells/dish of COS7 cells were cultured in 35-mm glass-bottom dishes (Mat Tek) and then transfected with various plasmids in a total amount of 0.5 μg/dish using SuperFect. For co expression studies, the total amount of vector added to each dish was equalized by the addition of empty vector. Sixteen to 24 hours after incubation in serum free GIT, the culture media were replaced with fresh GIT in the presence or absence of the DHT or FSK, and then the cells were observed with a Leica Corp. TSP-SP invert confocal laser scanning microscope (Leica Corp. Microsystems, Heidelberg, Germany), using a 100x, 1.4 numerical aperture PL APO oil immersion objective. Imaging for GFP and CFP was performed by excitation with the 488 nm, and 450 nm lines, respectively, from an argon laser, and the emissions were viewed through band passes ranging from 500 to 550 nm and from 470 to 500 nm, respectively, by band pass regulation with a Prism System (Leica Corp. Microsystems). For simultaneous imaging of multiple fluorescent proteins, the laser line was changed, and the band pass was further finely controlled so as not to overlap emissions. A three-dimensional imaging study was performed essentially in the same manner as previously reported [5]. Both the spatial distribution and calculations of the fluorescent proteins as a distinct volume were made possible by removing scattering background fluorescence and lens spherical aberrations and then by separating each particle.

RESULTS

Ligand induced subcellular translocation and transcriptional activation of AR

AR located in the cytoplasm in a homogenously diffuse manner in most cells in the absence of ligand (Figure 1A). Upon addition of DHT to the culture media, the nuclear receptor translocated to the nucleus where it further underwent compartmentalization so that it aggregated in distinct speckled foci (Figure 1B). A representative COS7 cell expressing AR-CFP in the absence and presence of 10^{-8} M of DHT are shown [7,8] (Figure 1A and Figure 1B). These nuclear translocation and foci formation processes were associated with activation of transcriptional activity of AR as monitored by ARE-Luc a promoter report containing specific AR response element (Figure 1C), and by MMTV-Luc, a reporter containing multiple NRs response element including that for AR (data not shown) .

Subnuclear distribution of CREB and transactovity

In a parallel experiment, we studied the subcelluar localization and transactivation of CREB, a TF of the GPCR-cAMP-PKA pathway. As shown by a representative COS7 cell expressing CREB-GFP, the TF located in the nucleus in a homogenously diffuse manner at baseline but reorganized to form foci upon stimulation with 10^{-6} M of FSK, a direct activator of adenylyl cyclase that turns on the cAMP-PKA pathway (Figure 2A and Figure 2B). This subnuclear reorganization process was associated with activation of CREB transcriptional activity as monitored by CRE-Luc a promoter report with specific CREB response element (Figure2C).

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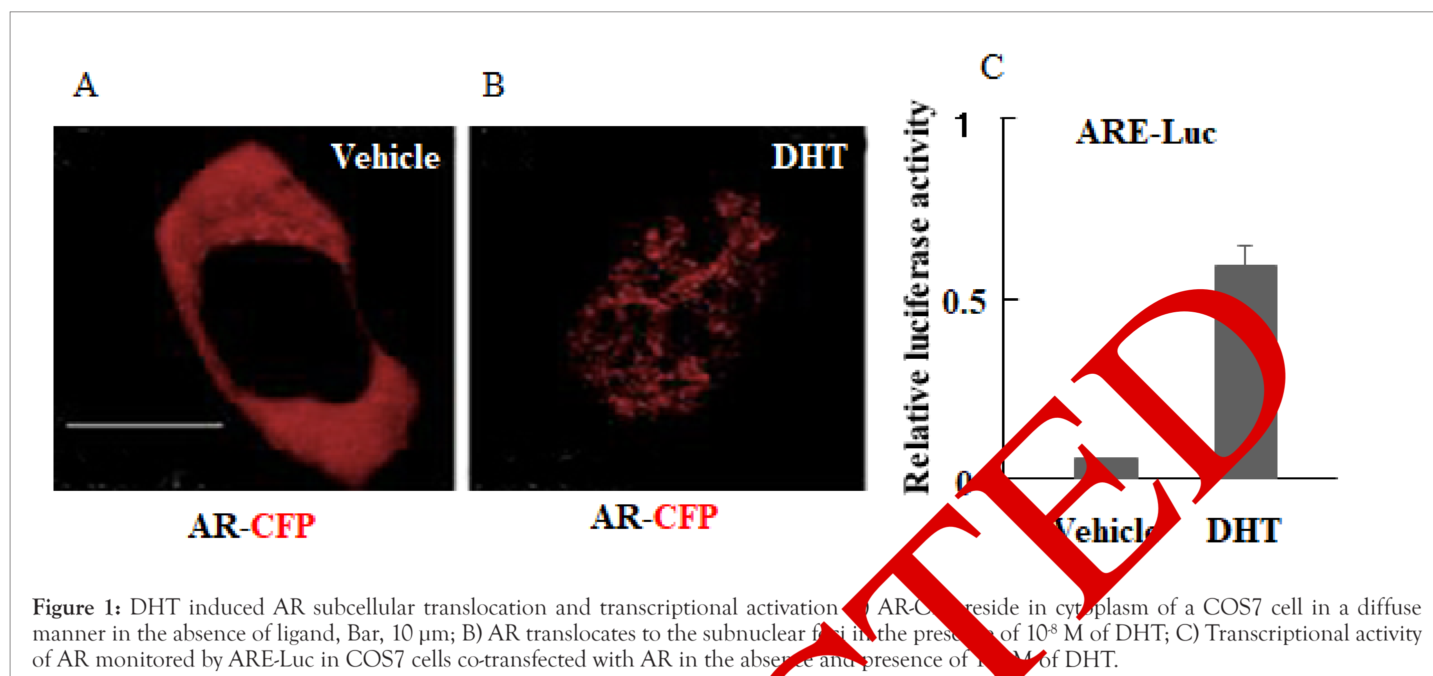

Figure 1: DHT induced AR subcellular translocation and transcriptional activation. A) AR-CFP reside in cytoplasm of a COS7 cell in a diffuse manner in the absence of ligand, Bar, 10 μm; B) AR translocates to the subnuclear foci in the presence of 10^{-8} M of DHT; C) Transcriptional activity of AR monitored by ARE-Luc in COS7 cells co-transfected with AR in the absence and presence of 10^{-8} M of DHT.

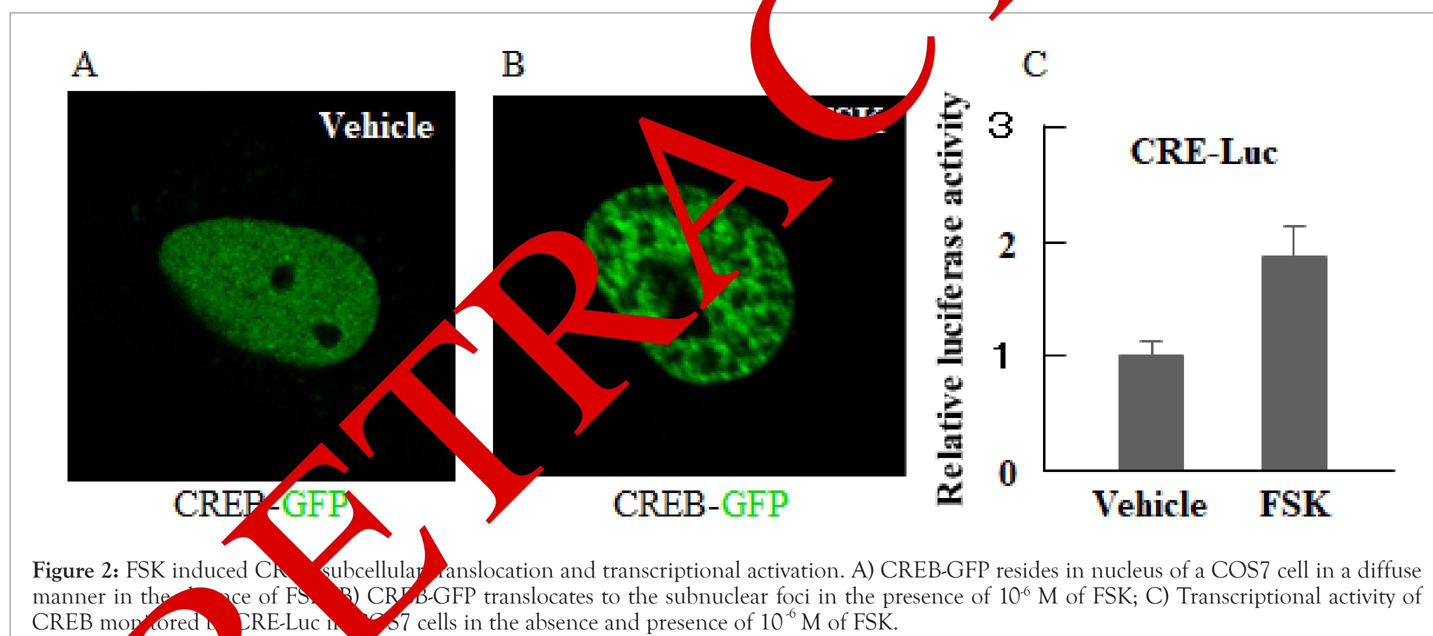

Figure 2: FSK induced CREB subcellular translocation and transcriptional activation. A) CREB-GFP resides in nucleus of a COS7 cell in a diffuse manner in the absence of FSK; B) CREB-GFP translocates to the subnuclear foci in the presence of 10^{-6} M of FSK; C) Transcriptional activity of CREB monitored by CRE-Luc in COS7 cells in the absence and presence of 10^{-6} M of FSK.

PKA induced subcellular reorganization and activation of AR

While most cells have AR located in the cytoplasm in the absence of androgen, around 20% of cells do have AR predominantly located in nucleus, which is likely due to a relative abundance of AR, in these cells, over the protein chaperones of heat shock proteins (HSPs), with the latter otherwise masks the nuclear localization signal (NLS) of AR and renders AR to reside in cytoplasm [9]. As shown by a representative cell expressing AR-GFP, AR located in the nucleus in this subset of cells in a diffuse manner at baseline (Figure 3A). Of interest, FSK, an activator of the cAMP-PKA pathway, reorganized AR to form foci in the absence of androgen (Figure 3B). Figure 3C showed that FSK also increased ARE-Luc activity in an AR dependent manner, suggesting PKA mediated induction of AR transactivation.

DHT induced subcellular reorganization of AR and CREB

In cells co-expressing both AR-CFP and CREB-GFP, AR resided in cytoplasm in a diffuse manner in most cells in the absence of ligand and this pattern was unchanged by FSK (Figure 4A and 4B). CREB localized diffusely in the nucleus at baseline but was translocated to Subnuclear foci in the presence of FSK, though the nuclear compartmentalization process appeared partially interfered with in the presence of AR than in the absence of the NR (Figure 4B). Of interest, DHT reorganized not only AR but also CREB to Subnuclear foci where both transcriptional factors colocalized (Figure 4C). The DHT induced AR-CREB foci remained unchanged with addition of FSK (Figure 4D). In a subset of cells where we were able to create 3D imaging [5,6,10] to count foci numbers, FSK was found unable to change the number of foci induced by DHT (301 ± 11 in cells treated with DHT only vs. 301 ± 11 in cells treated with DHT+FSK) (Figure 4).

Cross-regulations of transactivation between AR and CREB

DHT induced transactivation of AR, as monitored by ARE-Luc, was enhanced by FSK (Figure 5A). The FSK mediated transactivation of CREB, as monitored by CRE-Luc, was however suppressed by DHT in an AR dependent manner (Figure 5B).

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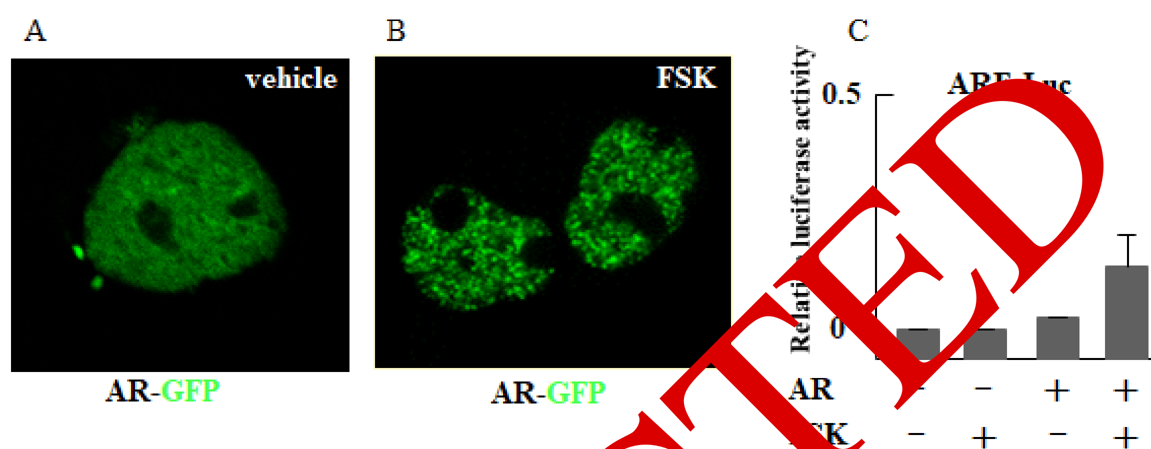

Figure 3: FSK induced AR subnuclear translocation and transcriptional activation in a small subset of cells. A) A representative COS7 where AR-GFP reside in nucleus in a diffuse manner in the absence of DHT; B) AR-GFP translocate to the Subnuclear foci in the presence of 10^{-6} M of FSK; C) Transcriptional activities of AR monitored by ARE-Luc in COS7 cells with or without AR co-expression, and in the absence and presence of 10^{-6} M of FSK.

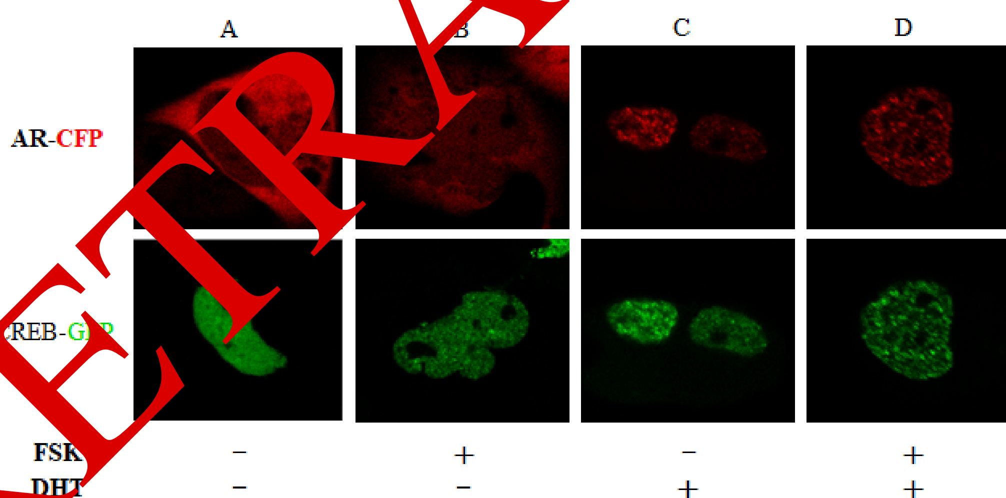

Figure 4: D[illegible] induced subcellular translocations of AR and CREB. A) A representative COS7 cell co-expressing AR-CFP and CREB-GFP in the absence of DHT or FSK. AR and CREB reside in cytoplasm and nucleus, respectively, both in a diffuse manner; B) Representative COS7 cells co-expressing AR-CFP and CREB-GFP were stimulated with FSK. CREB translocate to the subnuclear foci whereas AR remains in cytoplasm in a diffuse manner; C) Representative COS7 cells co-expressing AR-CFP and CREB-GFP were stimulated with DHT. Both AR and CREB translocate to the subnuclear foci where two TFs co localize; D) A representative COS7 cells co-expressing AR-CFP and CREB-GFP were stimulated with both DHT and FSK. Both AR and CREB translocate to the subnuclear foci where two TFs colocalize. The numbers of foci did not differ as compared to cells treated with DHT only (cells represented in Panel C).

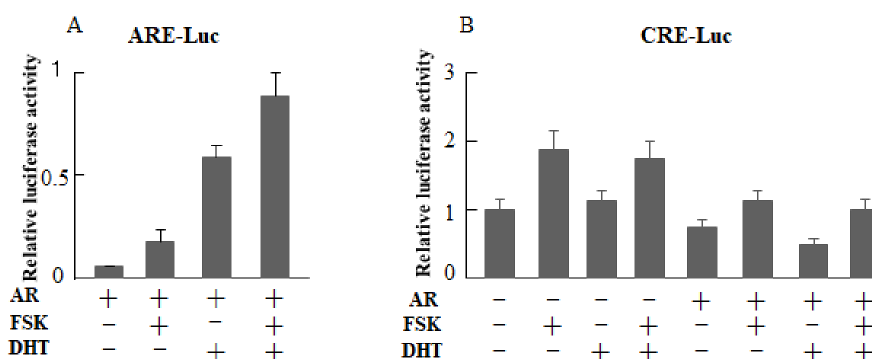

Figure 5: Bi-directional and differential cross-regulations of transcriptional activities of AR and CREB. A) Transcriptional activities of AR monitored by ARE-Luc in COS7 cells co-expressing AR in the presence of 10^{-6} M of FSK, 10^{-8} M of DHT or both; B) Transcriptional activities of CREB monitored by CRE-Luc in COS7 cells with or without AR co-expression, in the presence of 10^{-6} M of FSK, 10^{-8} M of DHT or both.

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DISCUSSION

NRs and GPCRs are two distinct principal signal transduction pathways by which eukaryotic cells response to external stimuli. While NRs rely binding of cognate ligands for functional regulation, GPCRs involve activation of specify intracellular kinases such as PKA. Both pathways ultimately relay signals to the nucleus culminating in modulation of gene expression.

It has been repeatedly shown that there are highly coordinated crosstalk/interactions between the two categories of signaling pathways, and that such cross-regulation leads to physiological and pathophysiological consequences. For instance, cAMP-PKA signaling is able to enhance the transactivation of the classic NR of glucocorticoid receptor (GR) [11] as well as orphan nuclear receptor of SF-1[12].

The cross-regulations could potentially occur at various levels of signal cascades that include expression levels of receptors, covalent modification (e.g., phosphorylation) of TFs, ligand-receptor affinities, receptor-co regulator interactions [12] as well as binding affinities between ligand-bound NR and its cognate response element on target DNA[11].

Lasor confocal microscopic studies of fluorescent proteins-tagged NRs allow direct observation of ligand induced dynamic intracellular translocation of NRs in living cells and have advanced our understandings of transcriptional regulation by NRs. One of the notable findings of such studies is that agonistic ligand-binding often induces a subnuclear compartmentalization process of NRs such that the receptors are redistributed into distinct speckles within the nucleus, a phenomenon often referred as "foci formation". AR, upon binding to DHT, forms 250-400 foci in the boundary region between euchromatin and heterochromatin [5,6,13]. Ligand induced subnuclear foci formation was also reported in cases of MR [14], ER [15,16] and vitamin D receptor [17]. The present study shows that CREB, a TF down stream of GPCRs signaling, also translocate to subnuclear foci, upon activation by cAMP-PKA. While the exact functional significances of this foci formation process are yet to be elucidated, available knowledge generally suggest that foci formation is associated with full transcriptional activity of NRs and foci compartments may represent the common sites for storage and/or assembly of various activated NRs (such as AR, ER, GR, SF1) along with the transcriptional coactivators (such as SRC-1, TIF2, and CBP) as proposed by the "common compartment hypothesis"[6]. NRs along with their cofactors can then dynamically cycle on and off in between foci compartments and the active transcription sites.

The results of the present study on AR and CREB subcellular translocation and transcriptional regulation suggest that the subnuclear foci may also provide geographic locations and mechanism whereby cross-regulations between NRs and GPCRs signals remain possible at the "final step" when TFs of either pathway are fully activated and poised to regulate target genes expression. As summarized in a model-of-action diagram shown in Figure. 6, activation of CREB by FSK can "drag" unliganded AR that's seen in nucleus of a minority of cells to subnuclear foci and renders AR partially active in terms of transactivation. DHT-induced AR foci formation is associated with translocation of CREB, in the absence of PKA activation, such that CREB colocalize with AR in the subnuclear foci, and this process is associated with enhanced AR transactivation. DHT-medicated AR transactivation is further enhanced by CREB in the presence of FSK, although the chemical does not appear alter foci numbers. While the effect of CREB on AR is stimulatory, the effect of the reverse direction appears suppressive. While DHT-bound AR does "drag" CREB to subnuclear foci in the absence of FSK, this is not associated with induction of CREB target promoter. FSK-induced CREB transactivation is dampened by DHT-bound AR. This "last step" cross-regulations between activated AR and CREB at the transactivation levels are therefore bi-directional and differential. They may have specific physiological and pathophysiological consequences under certain biological settings as exemplified by aforementioned two studies where PKA-CREB enhances AR-mediated gene transcription of PSA in prostate (cancer) cells[3] and androgen-AR signaling

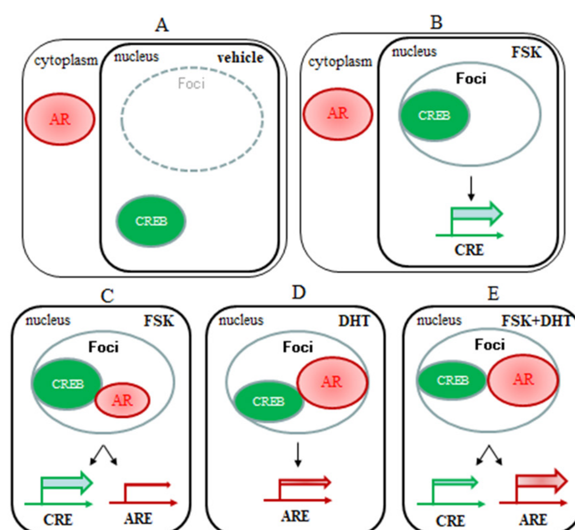

Figure 6: A model-of-action diagram. A) AR and CREB reside in cytoplasm and nucleus, respectively, both in a diffuse manner, at baseline. Both TFs are transcriptionally silent; B) Upon activation by cAMP-PKA, CREB translocate to Subnuclear foci. CREB is now transcriptionally fully active. AR, in most cells, remain in cytoplasm and transcriptionally silent; C) In a small subset of cells where AR resides diffusely in the nucleus in the absence of androgen, FSK translocate AR to subnuclear foci. CREB is transcriptionally active, and AR is transcriptionally partially active; D) In cells treated with DHT, both AR and CREB are translocated to subnuclear foci where they co localize. AR is transcriptionally fully active whereas CREB is transcriptionally silent; E) In cells treated with both DHT and FSK, both AR and CREB are translocated to subnuclear foci where they co localize. AR transcription is further enhanced as compared to that of without FSK (as seen in Panel D), whereas CREB transactivation is dampened as compared to that without DHT (as seen in Panel B).

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suppresses PKA-CREB mediated UCP1 expression in brown adipose tissue in male mice[4].

CONCLUSION

The present study shows that ligand-bound AR and activated CREB are translocated to the identical subnuclear foci where the two TFs are subjected to mutual and differential cross-regulations such that CREB enhances DHT mediated AR transactivation whereas ligand-bound AR suppressed PKA induced CREB transactivation. The cross-regulation between the TFs of classic NRs and GPCRs signaling pathways at transcriptional level may have physiological and pathophysiological consequences.

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CONFLICT OF INTEREST

All three authors testify that there is no conflict of interest and nothing relevant to current study to disclose.

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