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VIEWPOINT

Membrane Receptors for Steroid Hormones: Signal Transduction and Physiological Significance

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Abstract Membrane receptors for steroid hormones affect signaling pathways that modulate nuclear function, influence neuronal activity, ion flow, and the circulatory system. Indeed, 'new' steroid hormones have been identified by their interaction with membrane-initiated signaling systems. A brief summary of the FASEB Summer Research Conference devoted to these topics is presented in this mini-review. In addition, attendees of the meeting propose introduction of the following terminology: membrane-initiated steroid signaling (MISS) and nuclear-initiated steroid signaling (NISS) to replace more inaccurate terms in current use. J. Cell. Biochem. 88: 438–445, 2003. © 2003 Wiley-Liss, Inc.

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RECEPTOR SIGNALING, CROSSTALK WITH THE NUCLEUS, AND CELL DIFFERENTIATION

Of the many speakers in this session, the presentation on differentiation of leukemia cells by Dr. Kelly Meckling, University of Guelph, exemplified the topic under discussion. Lanotte et al. [1991] originally described the cell line NB4 as the only bonafide acute promyelocytic leukemia (APL) cell line available bearing the chromosomal translocation 15;17, typical of the majority of patients with APL. Retinoic acid is an effective treatment in APL inducing granulocytic differentiation of APL blasts and the NB4 cell line [Benoit et al., 2001]. NB4 cells are also capable of monocytic differentiation using combinations of 1α, 25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and PKC activators [Berry et al., 1996]. During the priming phase with 1,25-$(OH)_2D_3$ or related analogs, the major pathway of hormone action involves a membrane-initiated steroid signaling response, henceforth abbreviated MISS. Recent studies have focused on characterizing the signaling molecules involved in 1,25$(OH)_2D_3$ action and the action of other analogs of vitamin D with MISS activity. Cellular responses to 1,25$(OH)_2D_3$ that are necessary for differentiation include activation and nuclear translocation of PKC isoforms α and δ, phosphorylation of the inhibitory Kappa B binding protein (IκBα), degradation of IκBα via calpain and proteosome degradation pathways, and translocation of NFκB to the nucleus [Berry et al., 2002]. These processes are dependent on cytoplasmic Ca^{2+}, and can be mimicked by a variety of vitamin D analogs including MC903 and KH1060. The monocytic differentiation program involving 1,25$(OH)_2D_3$ and bryostatin induces predominantly a G1 arrest, with some cells arresting in G2. Typical regulators of this process include cdks 1, 2, and 4 and RBBP. Studies are ongoing to identify the upstream

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kinase responsible for vitamin D induced phosphorylation of IκBα and the membrane receptor initiating these events.

IDENTIFICATION OF NEW STEROID HORMONES ACTING THROUGH MEMBRANE RECEPTORS

Dr. Ilka Nemere, Utah State University, presented evidence for the endocrine actions of 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$]. The steroid 24,25$(OH)_2D_3$ is synthesized from 25-hydroxyvitamin D_3 as an alternative to the production of 1,25$(OH)_2D_3$. While many have held the view that 24,25$(OH)_2D_3$ represents an inactivation product to down-regulate 1,25$(OH)_2D_3$-stimulated intestinal calcium transport, a new hormonal role for 24,25$(OH)_2D_3$ is now apparent. Although a nuclear receptor for 24,25$(OH)_2D_3$ has not been identified to date, this metabolite is capable of inhibiting the rapid, MISS effects of 1,25$(OH)_2D_3$ [Nemere, 1999]. This prompted a search for a receptor for 24,25$(OH)_2D_3$ in chick intestinal epithelium, as well as efforts to characterize the binding activity and purify the protein. While the nuclear fraction contained only low levels of binding, the post-nuclear pellet containing basal-lateral membranes, Golgi, mitochondria, and lysosomes exhibited an enrichment in specific binding. The highest levels of binding in fractions resolved on Percoll gradients were evident in those enriched in lysosomal marker enzymes, followed by basal-lateral membrane fractions. Western analyses indicated that the binding activity was not due to contamination with the serum transport protein, DBP, in either lysosomal or basal-lateral membrane fractions. Further characterization of specific, saturable binding in the lysosomal fraction indicated an affinity ($K_d = 7$ nM) commensurate with circulating levels of steroid [Nemere et al., 2002]. Competition studies indicated an equivalent level of binding for both the 24R,25$(OH)_2D_3$ and 24S,25$(OH)_2D_3$ isomers, with significantly lower levels of competition by 25$(OH)D_3$ and 1,25$(OH)_2D_3$. The apparent high affinity of the binding activity for the 24S,25$(OH)_2D_3$ isomer was paralleled by its efficacy in the perfused duodenal loop for abolishing 1,25$(OH)_2D_3$-stimulated intestinal calcium transport. In keeping with the anti-hypercalcemic effect in the avian model system, Dr. Nemere reported that adaptation of freshwater (low calcium) fish to sea water (high calcium environment) was characterized by decreased levels of basal-lateral membrane receptors for 1,25$(OH)_2D_3$ in enterocytes and a concomitant increase in 24,25$(OH)_2D_3$ receptors.

Dr. Yanhai Yin of the Salk Institute for Biological Studies reported on MISS effects in plants by brassinosteroids (BR). BRs play important roles throughout plant growth and development. Plants defective in BR biosynthesis or perception display pleiotropic dwarf phenotypes characterized by impaired cell elongation and vascular differentiation and delayed senescence. BRs signal through a plasma membrane-localized, leucine-rich repeat (LRR) receptor kinase, BRI1 [Li and Chory, 1997]. Loss-of-function *bri1* mutants in *Arabidopsis* display BR-insensitive dwarf phenotypes. In addition, BRs specifically bind to a BRI1 complex and such binding results in BRI1 autophosphorylation [Wang et al., 2001]. These results indicate that BRI1 is a critical component of the BR receptor complex. By screening for *bri1* genetic suppressors and resistant mutant to a BR biosynthesis inhibitor, the Chory group identified two homologous proteins, BES1 and BZR1, that act as positive regulators downstream of BRI1 [Wang et al., 2002; Yin et al., 2002]. BES1 apparently transduces the BR signal from the membrane-bound BRI1 to the nucleus as the protein accumulates in the nucleus in response to BRs in a BRI1-dependent manner and activates BR target gene expression [Yin et al., 2002]. BES1 interacts with and is phosphorylated by BIN2, a glycogen synthase kinase-3 (GSK-3) implicated as a negative regulator in BR pathway. In addition, BR-induced BES1 accumulation is impaired in the gain-of-function *bin2-D* mutant, demonstrating that BIN2 negatively regulate BES1 protein levels in vivo. These results establish a signaling cascade for plant steroids with similarities to the Wnt pathway, in which signaling through cell surface receptors leads to inactivation of a GSK-3 allowing accumulation of a nuclear protein that regulates target gene expression [Yin et al., 2002].

STEROID HORMONES IN NEURONAL TISSUES

Inhibitory actions of glucocorticoids in neuronal tissue were reviewed by Dr. Jeffrey Tasker,

Tulane University. Adrenal glucocorticoids feed back onto the brain to inhibit the expression and secretion of hypothalamic hormones [Dallman et al., 1987] Glucocorticoid feedback appears to be mediated by actions at different central targets, either indirectly via actions on the hippocampus or directly at the levels of the hypothalamus and pituitary [Herman et al., 1996]. In addition to NISS glucocorticoid effects mediated by intracellular steroid receptors, evidence for MISS glucocorticoid actions through plasma membrane receptors has accumulated in recent years [Suyemitsu and Terayama, 1975; Harrison et al., 1979; Orchinik et al., 1991]. Potential MISS effects of glucocorticoids on the synaptic activity of rat paraventricular nucleus (PVN) neurons in coronal slices of the hypothalamus were studied using whole-cell patch-clamp recordings. Glucocorticoids elicited a rapid (3–5 min), dose-dependent reduction in excitatory glutamatergic synaptic inputs to PVN neurons, with a threshold concentration of 1–10 nM. This effect is not dependent on activation of type I or type II corticosteroid receptors and appears to be mediated by an extracellular membrane receptor, since it was not abolished by conjugating the glucocorticoid to bovine serum albumin (BSA), and it was not reproduced with intracellular application of glucocorticoids. It is dependent on activation of a post-synaptic G-protein dependent mechanism, since blocking specifically post-synaptic G protein activity abolishes the effect. Thus, glucocorticoids activate a post-synaptic G protein-dependent pathway to cause a pre-synaptic suppression of glutamate release, which implicated retrograde signal transmission. Blockade or saturation of CB1 and CB2 cannabinoid receptors abolished the glucocorticoid effect on pre-synaptic glutamate release, indicating that it was mediated by a retrograde endocannabinoid messenger. These findings suggest that rapid glucocorticoid actions in the hypothalamus are mediated by activation of G protein-coupled membrane receptors and the resulting retrograde release of an endocannabinoid, which leads to suppression of excitatory glutamate inputs to PVN parvocellular neurons.

Dr. Cheryl Watson, University of Texas Medical Branch, summarized data from her laboratory on the identification of ERα as a membrane-resident steroid receptor [Pappas et al., 1994] in her various experimental systems including pituitary tumor cells (GH3/B6), breast cancer cells (MCF-7), fetal hippocampal cells, and pheochromocytoma cells (PC-12). A membrane-associated version of ERα has been identified using nine different antibodies and by antisense knock-down experiments [Watson et al., 2002]. A variety of rapid responses to estradiol and xenoestrogens have been documented, including prolactin release, cellular calcium elevation [Watson et al., 1999], MAP kinase activation, and cell shape changes leading to detachment from the growth substrate [Campbell et al., 2002]. Immunocytochemistry techniques, which utilize non-permeabilizing fixation techniques combined with confocal microscopy, further demonstrate the position of the receptor at the outer face of cell membranes as well as its aggregated presentation. Quantitative plate immunoassays measuring membrane versus intracellular receptors under a variety of regulatory conditions demonstrated differential changes in the two receptor subpopulations [Campbell et al., 2002]. For example, the membrane population of ERα receptors decline with increasing cell density (transitioning just as cells begin contact) while the nuclear ERα receptors increase their numbers under these conditions. Therefore, the study of these two receptor populations in tandem is revealing their similarities in structure, but their very different cellular presentation and regulatory responses.

In some cells, the membrane receptor has been found to recognize ligand-bound serum transport proteins for a steroid. One such system utilizing sex steroid hormone binding globulin was described by Dr. Jack Caldwell, University of Illinois Medical College. Sex hormone binding globulin (SHBG) is a steroid carrier that has plasma membrane receptors in the prostate and uterus. SHBG infusions into the medial preoptic area facilitated sexual receptivity in females whether it was coupled with estradiol or not. SHBG is produced in the PVN, supraoptic nucleus, and several other areas that also have the peptide oxytocin. Therefore, immunocytochemistry was used to analyze whether SHBG was co-localized with oxytocin. In every brain region that contained oxytocinergic cells, some portion of those cells containing SHBG were found. This number was increased by colchicine treatment, suggesting that SHBG is rapidly transported out of perikarya to processes. SHBG co-localized with oxytocin in Herring bodies of the posterior pituitary

suggesting that it might be co-released with oxytocin. SHBG immunoreactive processes with varicosities were observed near oxytocin cell bodies in the PVN as well as SHBG immunoreactive pre-synaptic elements opposing oxytocin cells using electron micrography, suggesting that SHBG is released onto oxytocin cells. Brain homogenates containing the PVN are stimulated to release oxytocin immediately upon exposure to estradiol conjugated to BSA (E-BSA; but not to free estradiol). Dihydrotestosterone and 3α-diol, 5α-reduced steroids known to block SHBG receptors, were found to block this E-BSA-stimulated release of oxytocin. Therefore, it appears that SHBG release within the preoptic area and hypothalamus stimulates release of oxytocin from the same or different neurons. As occurs with cytoplasmic estradiol receptors in brain, in vivo estradiol treatment virtually shuts down SHBG mRNA levels as measured by RT-PCR in the medial preoptic area, PVN, and other brain areas. This suggests that SHBG production is sensitive to endocrine state and responds similarly to other steroid-binding proteins. Conjugated steroids including estradiol-BSA (E-BSA) and progesterone-BSA (P-BSA) have a high density of binding sites in the same brain areas where SHBG is made and may be released. SHBG displaces bound radiolabeled BSA-conjugated steroids suggesting that some portions of those sites are SHBG receptors. Dr. Caldwell's current model is one in which SHBG binding sites in brain are part of a larger membrane-associated steroid receptor that interacts with oxytocin receptors via a common G-protein.

STEROID MEMBRANE RECEPTORS AND ION FLOW

Numerous steroid hormones have been found to rapidly influence ion channel activity, including sex steroids. Ion translocation and steroid receptors in the plasma membrane of bone cells were evaluated by Dr. Carol Gay, Pennsylvania State University. Osteoblasts and osteoclasts respond in a rapid manner to gonadal steroids following binding at the plasma membrane. Flow cytometry has revealed that both testosterone (T) and 17α-estradiol (E) conjugated to BSA bind to osteoblasts derived from 3-week-old male chickens in approximately equal amounts; both T-BSA and E-BSA elicited Ca^{++} influx that was blocked by verapamil-sensitive calcium channels [Armen and Gay, 2000]. This is distinct from studies on adult human osteoblasts, in which rapid activation of creatine kinase was gender specific; specifically, female-derived cells responded to estradiol and male-derived cells responded to testosterone [Katzburg et al., 2001]. The differences may be related to donor age or to the signaling pathway investigated. Osteoclasts, also derived from 3-week-old male chickens, respond to gonadal steroids in a distinctly different manner from osteoblasts. E-BSA did not elicit a rise in cytosolic Ca^{++}. However, osteoclast membrane potential rapidly depolarized when treated with E-BSA [Brubaker and Gay, 1999a]. By using an array of inhibitors, it was shown that K^+ influx through inward directed K^+ channels supported the depolarization. Another rapid response of osteoclasts to E-BSA is the phosphorylation and translocation of Src kinase to the plasma membrane, a process dependent on intact actin filaments [Brubaker and Gay, 1999b]. Osteoclast responses to T-BSA have not yet been examined.

Thyroid hormones are key regulators of metabolism and development. Dr. Paul Davis, Albany Medical College, reported that, despite distinctive structures and biological effects mediated by discrete nuclear receptor proteins, thyroid hormone, and steroids are now understood to utilize similar mechanisms in their membrane-initiated actions. For example, steroids and thyroid hormone both activate the mitogen-activated protein kinase (MAPK) signal transduction cascade, cause nuclear translocation of MAPK, and MAPK-dependent serine phosphorylation of their respective nuclear superfamily receptors. These are examples of interfaces between NISS and MISS actions of the hormones. Both families of hormones may increase $[Ca^{2+}]_i$, presumptively as an antecedent to MAPK activation via protein kinase C. Both families of hormones can promote intracellular trafficking of proteins and both can influence the activities of plasma membrane homeostatic transport systems, such as the Na^+/H^+ antiporter. Among the other MISS actions of thyroid hormone are those on (cell surface) Na^+ current and inward-rectifying K^+ current, plasma membrane Ca^{2+}-ATPase activity, solubility of actin, and mitochondrial respiration, perhaps via direct interaction of the hormone with a subunit of mitochondrial cytochrome oxidase. In addition to its action to

serine phosphorylate nuclear thyroid hormone receptor (TRβ1), causing shedding of corepressor proteins, thyroxine (T4)-directed MAPK also causes serine phosphorylation of p53 and STAT nucleoproteins. These phosphorylation steps alter the activities of the nucleoproteins. Thyroid hormone has also been shown to have MISS effects on heart vascular smooth muscle and to have acute cardiovascular effects in intact human subjects. However, ambient thyroid hormone levels are relatively stable, and it is proposed that MISS effects of the hormone primarily contribute to the setpoints of activity (basal activities) of the membrane systems and nucleoproteins on which the hormone has been shown to have effects.

Some of the first studies of rapid responses to steroid hormones were performed on frog skin, which showed rapid (<1 min) activation of basolateral K^+ recycling and Na/H exchange following exposure to aldosterone. These rapid MISS effects are also observed in other Na^+ and Cl^- transporting epithelia such as human sweat gland and colon in response to physiological concentrations of aldosterone and estradiol. Dr. Brian Harvey, Royal College of Surgeons in Ireland, described the signal transduction pathways (Ca^{2+}, PKC, PKA, and MAP kinases) involved in rapid responses to aldosterone and estradiol and discussed their physiological impact. While the molecular identity of the MISS receptor(s) still remains elusive, there is strong evidence for direct activation of specific PKC isoforms by aldosterone and estradiol. For example, aldosterone directly stimulates the activity of the purified recombinant PKCα isoform (but not PKCδ, PKCϵ, and PKCζ) in a cell-free assay system, whereas estradiol directly stimulates the activity of both PKCδ and PKCα (but not PKCϵ and PKCζ). Evidence for other MISS receptor candidates such as a Gαs-coupled receptor were discussed. The MISS response to estradiol in distal colon appears to reduce the capacity for secretion (by inhibiting Ca^{2+}-dependent K^+ channels in crypt cells). This conclusion is borne out by experiments in rat and human colon showing that physiological concentrations of estradiol (0.1–10 nM) can reduce both the basal and secretagogue-induced Cl^- secretion (e.g., cholera toxin, forskolin, and carbachol). The antisecretory effect of estradiol is sensitive to PKC inhibition and is female gender specific.

Thyroid hormones are able to alter cardiac electrophysiological properties in animals and humans. However, the mechanism of these effects is unclear. Dr. Luyi Sen's group, University of California, Los Angeles, have previously reported that acute 3,5,3′-triiode-L-thyronine (T_3) exposure could shorten APD and enhance the whole-cell I_{K1} current in guinea-pig ventricular myocytes by extranuclear mechanisms. T_3 enhances the single I_{K1} current with the increment in P_o, which mainly results from shortening of inter-burst duration without any change in burst behavior. The shortened inter-burst duration by T_3 is mainly due to reduced probability of the C_1 state (P_1), which results from acceleration of the transition from the C_1 to the C_2 states. The findings indicate that the enhanced I_{K1} by T_3 might be one of the candidates for shortened APD in hyperthyroidism. Recently, acute effects of T_3 on cardiac sodium channel (I_{Na}) properties in adult guinea-pig ventricular myocytes were evaluated using whole-cell, cell-attached, and inside-out patch recording techniques. T_3 (1 nM) acutely increased whole-cell I_{Na} current and shifted the steady-state I_{Na} inactivation curve in a dose-dependent manner. This effect was reproduced by 3,5,3′-triiodothyroacetic acid (TAA), and blocked by 3′,5′,3-L-trioiodothyronine (rT_3). When the pipette solution contained 100 μM GTP or GTPγS, the effects of T_3 on the whole-cell I_{Na} were increased twofold to threefold. This effect was almost completely abolished by pertusis toxin pre-incubation. In cell-attached patches, T_3 increased the open probability of single I_{Na} channel by reducing the null probability without changing the mean open time and the number of available channels. In the inside-out patch configuration, the increase in single I_{Na} channel current was observed only within 30 s after T_3 exposure while GTPγS was present. This effect was ten times faster than that was observed in whole cell and cell-attached patches, and could be completely washed out. Without GTPγS, 10 nM T_3 only slightly increased the channel open probability by increasing rate constant k_{CO}, and reducing the null probability. GTPγS exposure only increased the number of functional channels. T_3 (10 nM) and GTPγS (10 μM) synergistically enhanced the channel open probability 5.8 ± 0.5-fold by increasing k_{CO}, decreasing k_{OI}, and greatly reducing P_N. These results demonstrate that T_3 acts on the cytosolic side of the ventricular

myocyte membrane and acutely activates sodium channels. Pertussis toxin sensitive G protein modulation greatly magnifies the T_3 effects on the channel kinetics and null probability, and thereby increases the channel open probability. The synergistic interaction between the pathophysiologically increased G protein and T_3 levels may have a profound impact on Na^+ channel kinetics. Especially, this synergy significantly slows Na^+ channel inactivation, increases fast inward Na^+ current, and consequently increases the membrane excitability that may be the co-factor of ventricular arrhythmias in hyperthyroid patients with hypertrophy, ischemia, and/or heart failure.

STEROID MEMBRANE RECEPTORS AND THE CIRCULATORY SYSTEM

The effect of steroids and their metabolites on intracellular free calcium ($[Ca^{2+}]_i$) was examined in human platelets by Dr. Peter Blackmore, Eastern Virginia Medical School. Progesterone metabolites were shown to increase ($[Ca^{2+}]_i$) within several seconds, and maximal effects were observed within 1–2 min. Pregnanediol was the most effective steroid metabolite at increasing ($[Ca^{2+}]_i$), isopregnanediol was less effective. Pregnanolone was the next most potent stimulator of ($[Ca^{2+}]_i$). Pregnanetriol was without effect, indicating that substitutions on the D ring have a major influence on activity. Dose response experiments showed that 0.1 μM pregnanolone produced significant increases in ($[Ca^{2+}]_i$), with maximum increases in ($[Ca^{2+}]_i$) being observed with 10 μM pregnenolone. Low concentrations of pregnanolone potentiated the effects of low concentrations of thrombin to increase ($[Ca^{2+}]_i$), indicating that both agonists increase ($[Ca^{2+}]_i$) by different mechanisms. The effect of pregnanediol to increase ($[Ca^{2+}]_i$) was inhibited by the phospholipase C inhibitor U73122. The src inhibitors PP1 and PP2 inhibited the effects of pregnanediol and collagen to increase ($[Ca^{2+}]_i$), whereas the inactive inhibitor PP3 was without effect. It was concluded that progesterone metabolites increase ($[Ca^{2+}]_i$) by a mechanism that is similar to that of collagen in human platelets.

Although the exact role(s) that estrogen plays on the vascular wall remain incompletely understood, the gender difference in cardiovascular disease incidence between males and age-matched pre-menopausal women, and the plethora of "vasculoprotective" animal data in vivo, support that estrogen does contribute to cardiovascular health in women. The endothelium is at least one major target of estrogen. The laboratory of Dr. Jeffrey Bender, Yale University, has been interested in the mechanisms by which 17beta-estradiol (E2) activates endothelial nitric oxide synthase (eNOS) and consequently augments basal NO release. In earlier studies of such, it became clear that these activation responses occur rapidly and do not require induced transcription. This led to the hypothesis that membrane-localized receptors are the primary signal transducing elements for these events. In fact, shorter forms of ERα (ER-46 kDa) may be the predominant form in human endothelial cells (EC), and that this form is fully capable of mediating eNOS activation responses to E2. Immunofluorescence data demonstrate coincident expression of membrane ERs and membrane-binding sites for E2. Furthermore, a critical signal transduction cascade, requiring PI-3 kinase-dependent AKT activation (with consequent eNOS phosphorylation/activation), is initiated by membrane-impermeant estrogen and blocked by conventional ER antagonists. Finally, new and preliminary data support that additional non-receptor tyrosine kinases facilitate the E2-induced heterocomplex assembly required for signal propagation in EC.

SUBCELLULAR FATE OF STEROID MEMBRANE RECEPTORS

Dr. Bahiru Gametchu, Medical college of Wisconsin, presented data that the membrane glucocorticoid receptor is a modified version of the nuclear receptor, having previously demonstrated that multiple GR antibodies recognize both forms, and also that they arise from the same coding sequence. His presentation compared the expression of mGRs in several wild type (WT) and multiple mutant mouse lymphoid cell lines, grouped according to previous descriptions of their intracellular GR defects. A variety of immunological and biochemical techniques characterized the quality and quantity of mGR expression in each cell line, compared to functional consequences (glucocorticoid-induced apoptosis). Levels of mGR were shown to be four to ten times higher in the WT and mGR-enriched (mGR++) S-49 cells,

compared to several mutant cell groups which either lacked mGR or expressed negligible amounts. Glucocorticoid sensitivity assays showed a good correlation between the quantity and quality of mGR and the ability of dexamethasone to lyse these lymphoid cancer cells. The level of mGR also predicted the MAP kinase (ERK and JNK) activation responses of the cells, linking a rapid signaling response to the presence of membrane receptor. Exposure of mGR-enriched lysis-sensitive cells (but not mGR-less and lysis resistant cells) to dexamethasone-coupled BSA also evoked a very rapid (5–10 min) activation of MAP kinases and a cAMP increase. These data demonstrate a specific mechanistic linkage between the mGR, rapid signal generation, and the therapeutic apoptotic response in model lymphoma cell systems.

INTEGRATION OF SIGNALING PATHWAYS

In discussing current findings on membrane-associated estrogen receptors (ER) in breast cancer, Dr. Diana Marquez, UCLA School of Medicine, noted that ER occur in about 75% of breast cancers at diagnosis. These receptors are present in nuclear as well as membrane-associated sites in the cancer cell. Increasing evidence suggests that membrane signaling induced by ER may participate in the regulation of cell growth. Using controlled cell homogenization and fractionation of MCF-7 human breast cancer cells, the bulk of specific, high affinity estradiol-17β (E2) binding is found in nuclear fractions. However, a significant portion of specific high-affinity E2 binding is also enriched in plasma membranes. Two bands of 67 and 46 kDa can be detected using labeled estrogen or a monoclonal antibody directed against the ligand-binding domain of the nuclear ER-α (ERAb). In addition, estrogen-induced growth of MCF-7 cells in vitro correlates with acute activation of MAPK and Akt kinase signaling. Treatment of MCF-7 cells with ERAb blocked estrogen-induced growth of MCF-7 cells in vitro and rapid activation of MAPK and Akt signaling induced by E2. This effect was also observed in vivo, wherein ERAb was able to inhibit E2-induced growth of breast cancer tumor xenografts. Thus, membrane-associated forms of ER contribute to growth regulation of breast cancer cells and tumors. Further, Dr. Marquez presented evidence on the development of new treatments directed to alternate pathways of estrogen action in the cell in order to provide complementary or more effective therapies in breast cancer. A number of steroidal antiestrogens were designed to interact with different signaling pathways mediated by either nuclear or membrane forms of ER. The concept of estrogen acting via both nuclear and membrane-associated receptors presents a new challenge and a unique opportunity for the development of new therapies. Anti-hormone therapies that differentiate between nuclear and membrane-associated forms of ER may be important for future breast cancer treatment.

In previous work, the laboratory of Dr. Dalia Somjen, Tel Aviv Sourasky Medical Center, reported that estrogen treatment of cultured human vascular smooth muscle cells induces a biphasic effect on DNA synthesis, i.e., stimulation at low concentrations and inhibition at high concentrations. Additionally, estrogen increased the specific activity of creatine kinase in both vascular cell types, as well as in human cultured bone cells. Dr. Somjen's presentation focused on a study designed to determine whether or not some of these effects are exerted, at least in part, via membrane-associated binding sites, using native as well as novel protein-bound, membrane-impermeant estrogen complexes. Dr. Somjen assessed changes of DNA synthesis and creatine kinase after treatment with estradiol-17β, estradiol-17β-6-carboxy methyl oxime conjugated to BSA; 6-carboxymethyl genistein or 6-carboxymethyll genistein bound to the high molecular protein keyhole limpet hemocyanin; and 7-(O)-carboxy methyl daidzein or 7-(O)-carboxy methy daidzein linked to keyhole limpet hemocyanin. High concentrations of either estradiol or estradiol-BSA inhibited vascular smooth muscle cell proliferation (-39 ± 28 vs. $-32 \pm 15\%$). The phytoestrogen derivatives, 6-carboxy methyl genistein and 6-carboxymethy daidzein, as well as their respective conjugates with keyhole limpet hemocyanin, dose-dependently stimulated DNA synthesis in VSMC by (66 ± 2, 100 ± 12, 66 ± 6, and $41 \pm 8\%$ at 300 nM, respectively). In contrast, all forms of protein-bound hormones were unable to stimulate CK specific activity in VSMC as well as in human osteoblasts. Using the membrane-impermeant ligand estradiol-BSA linked to Eu, a binding assay for specific membrane binding sites for estradiol in VSMC and in female bone cells was developed. This binding is not competed by

Raloxifene, unlike nuclear binding of ligand, and it is modulated by vitamin D metabolites or hyperglycemia in both cell types. Finally, using the membrane impermeant ligand estradiol-BSA linked to the fluorescent dye Cy3.5, the presence of membrane binding sites for estradiol in VSMC and in female bone cells was demonstrated directly. Hence, some of the biological effects of estradiol in VSMC or bone cells are apparently exerted by membrane binding sites for estradiol and do not require intracellular entry of estradiol via the classical nuclear receptor route.

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